1-1-2002

Seed transmission of Fusarium species and systemic infection in maize

Amy Lynette Wilke
Iowa State University

Seed transmission of *Fusarium* species and systemic infection in maize

by

Amy Lynette Wilke

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Plant Pathology

Program of Study Committee:
Gary P. Munkvold (Major Professor)
Charlotte R. Bronson
Adriana Tomas
Manjit Misra

Iowa State University

Ames, Iowa

2002

Graduate College
Iowa State University

This is to certify that the master's thesis of

Amy Lynette Wilke

has met the thesis requirements of Iowa State University

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION

The genus *Fusarium* is a cosmopolitan group of fungi that cause a wide range of plant diseases. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon), *F. subglutinans* (Wollenw & Reinking) Nelson, Toussoun, and Marasas, and *F. proliferatum* (Matsushima) Nirenberg cause root rot, stalk rot, ear rot, and seedling blight of maize (*Zea mays* L.) (Smith and White 1988). These three species are among the most common pathogens associated with maize, and they are responsible for production of mycotoxins both in diseased and symptomless infected tissues. Mycotoxins have detrimental effects on animals and humans and are a problem when they enter the food chain. In order to reduce the risk of mycotoxin contamination of grain, it is important to understand sources of inoculum and infection pathways of these *Fusarium* species.

Taxonomy: *Fusarium* Section *Liseola*

Fusarium moniliforme usually has been reported as the most common species infecting maize. But because of disagreement and changes in taxonomy in this genus, it has not always been certain whether results reported for *F. moniliforme* refer to a single species or a group of species. In 1935, Wollenweber and Reinking developed a taxonomic scheme for the genus *Fusarium* that recognized 65 species, 55 varieties, and 22 forms that were arranged into 16 sections (Burgess et al. in press). This consolidated the approximately 1,000 previously described species of *Fusaria* by using distinct morphological characteristics and eliminating synonyms. Since this time, many other taxonomic systems have been

suggested but most have followed this general structure (Burgess et al. in press, Nelson et al. 1983). Currently, 12 of Wollenweber and Reinking's 16 sections are still being used.

Further discussion of the sections will focus on taxonomic development in the section *Liseola* and the species included in the section. In the 1940's, Snyder and Hansen consolidated the 65 species that Wollenweber and Reinking recognized to nine species; thus condensing the section *Liseola* to one species named *F. moniliforme* Sheld. emend. Snyd. & Hans. (Nelson 1991). Since then the taxonomic status of *F. moniliforme* has been changed several times to separate the distinct species (including *F. subglutinans* and *F. proliferatum*) that were lumped together under this name. In 1971, Booth followed Wollenweber by distinguishing *F. subglutinans* as a variety of *F. moniliforme* and in 1976, Nirenberg identified *F. proliferatum* as a separate species. In 1983, Nelson et al. placed four species in the section Liseola: *F. moniliforme* Sheldon, *F. subglutinans* (Wollenw & Reinking) Nelson, Toussoun, and Marasas, *F. proliferatum* (Matsushima) Nirenberg, and *F. anthophilum* (A. Braun) Wollenw. At this time, Nelson et al. (1983) recognized *F. subglutinans* as a distinct species from *F. moniliforme*. They also acknowledged that the species *F. moniliforme* had previously been described as *F. verticillioides* by Nirenberg in 1976 and listed it as a synonym. *F. verticillioides* has recently become accepted as the valid name for this species.

Nelson et al. (1983) grouped *F. verticillioides* (synonym *F. moniliforme*), *F. subglutinans*, and *F. proliferatum* together into a section based on morphological characteristics. All three species produce macroconidia from monophialides on branched conidiophores in sporodochia and produce microconidia from monophialides on hyphae. The formation of microconidia differs among the three species. *F. subglutinans* forms microconidia in false-heads from polyphialides and monophialides. *F. verticillioides* and *F.*

proliferatum produce microconidia in chains from monophialides on branched and unbranched conidiophores and from monophialides formed directly on the hyphae. The chains of microconidia produced by *F. verticillioides* are usually longer than the chains of microconidia produced by *F. proliferatum*. *F. proliferatum* also has polyphialides. Chlamydospores are absent in all three species (Nelson et al. 1983). Burgess et al. (in press) followed a similar system as Nelson for identifying the different species.

F. moniliforme, *F. subglutinans*, and *F. proliferatum* also were assigned the same teleomorph, *Gibberella fujikuroi* (Kuhlman 1982). Leslie (1991) divided *G. fujikuroi* into mating populations (A-F), which corresponded to six anamorphs (Table 1). More recently changes have been made to rename and divide these species again based on gene sequences (O'Donnell et al. 1998). Additional mating populations and anamorph names have been added (Klaasen and Nelson 1996, O'Donnell 1996).

Table 1. Anamorphs, teleomorphs, and mating populations of *G. fujikuroi* complex according to several authors.

Mating Population (Leslie 1991)	Anamorph (Nelson et al. 1983)	Teleomorph (Nelson et al. 1983)	Anamorph (Burgess et al. in press)	Teleomorph (Burgess et al. in press)
A	*F. moniliforme*	*G. fujikuroi*	*F. verticillioides*	*G. moniliformis*
B	*F. subglutinans*	*G. subglutinans*	*F. sacchari*	Undescribed
C	*F. proliferatum*	Undescribed	*F. fujikuroi*	*G. fujikuroi*
D	*F. proliferatum*	Undescribed	*F. proliferatum*	*G. intermedia*
E	*F. subglutinans*	*G. subglutinans*	*F. subglutinans*	*G. subglutinans*
F	*F. moniliforme*	*G. fujikuroi*	*F. thapsinum*	*G. thapsina*

Impacts on Maize

Infection and colonization of maize by *Fusarium* species can result in ear rot, stalk rot, root rot, and seedling blight. *F. verticillioides*, *F. subglutinans*, and *F. proliferatum* are causal agents of Fusarium ear rot (Vigier et al. 1997). This ear rot is distinguished from

others by its whitish to lavender fungal growth on randomly infected or insect-damaged kernels (Payne 1999). Symptoms also include a starburst effect on the kernels that consists of white streaks in the pericarp where the fungus has radiated out from the point of silk attachment (Payne 1999).

Stalk rot is caused by a combination of fungi including several species of *Fusarium*, including *F. verticillioides, F. subglutinans*, *F. proliferatum*, and *F. graminearum* (Kingsland and Wernham 1962, White 1999). After pollination most stalks are infected with one or more of these species but infection alone does not determine if stalk rot will occur. The largest contributing factor for stalk rot is plant stress (Kommedahl et al. 1979). Symptoms of stalk rot are decay of the stalk pith, premature death, breakage, and lodging of the plants. Lodging is the result of decaying structural components of the stalk, but not the rind or vascular bundles (Foley 1968). Stalk infection by *Fusarium* species can be symptomless (Foley 1962, Sumner 1968).

Fusarium root rot can be caused by many *Fusarium* species, including *F. verticillioides* and *F. subglutinans* (Munkvold and Leslie 1999). Symptoms include reduced root length and branching, brownish to black discoloration, and rotting (Futrell and Kilgore 1969).

Research on seedling blight has been limited primarily to *F. verticillioides* (Yates et al. 1997) and *F. graminearum* (Kabeere et al. 1997). Kernels infected with either species can produce seedlings with infected roots, mesocotyls, and stems (Foley 1962, Sumner 1968). Seedlings may also fail to emerge (Chelkowski 1989).

Infection and colonization of maize tissues may result in the production of secondary metabolites called mycotoxins. Mycotoxins can have toxic effects on plants, animals, and

humans. Some of the toxins produced by *F. verticillioides*, *F. subglutinans* and *F. proliferatum* are beauvericin, fumonisins, fusaric acid, fusaproliferin, and moniliformin. Beauvericin and fusaproliferin are both produced by *F. subglutinans* and *F. proliferatum*. Both mycotoxins have toxic effects on brine shrimp, human cells, and insects (Di Paola et al. 1994, Logreico et al. 1993, Logrieco et al. 1996). Fusaproliferin has been proven to have teratogenic effects on chicken embryos (Ritieni et al. 1997). Fumonisins are produced by *F. verticillioides* and *F. proliferatum* (Marasas et al. 2000). FB_1, FB_2, and FB_3 are the most commonly found fumonisins; several others (A_1, A_2, and C_1) have been described but are rarely found (Marasas et al. 2000). Fumonisins have been proven to cause leukoencephalomalacia (LEM) in horses (Kellerman et al. 1990), porcine pulmonary edema (PPE) in swine (Harrison et al. 1990), and are associated with esophogeal cancer in humans (Rheeder et al. 1992). Fusaric acid also is produced by *F. verticillioides*. This mycotoxin affects insects (Dowd 1988), mice, and swine. *F. subglutinans* and *F. proliferatum* also can produce moniliformin (Chelkowski et al. 1990). Moniliformin is toxic to chicks, ducklings, mice, and rats (Kriek et al. 1977) (Table 2).

Table 2. Mycotoxins produced by *F. verticillioides*, *F. subglutinans*, and *F. proliferatum* and the organisms that are affected.

Mycotoxin	Fusarium Species	Organism Affected
Beauvericin	*F. subglutinans* (Logrieco 1993) and *F. proliferatum* (Munkvold et al. 1998)	brine shrimp (Logrieco et al. 1993)
		human cells (Di Paola et al. 1994)
		insects (Grove and Pople 1980)
		murine cells (Ojcious et al. 1991)
Fumonisins (FB_1, FB_2, and FB_3)	*F. verticillioides* and *F. proliferatum* (Marasas et al. 2000)	horses (LEM) (Kellerman et al. 1990)
		humans (Rheeder et al. 1992)
		rats (Gelderblom et al. 1991)
		swine (PPE) (Harrison et al. 1990)
Fusaric Acid	*F. verticillioides*	insects (Dowd 1988)
		mice
		swine
Fusaproliferin	*F. subglutinans* and *F. proliferatum* (Munkvold et al. 1998)	brine shrimp (Logrieco et al. 1996)
		chicken embryos (Ritieni et al. 1997)
		IARC/LCL 171 human B Lymphocytes (Logrieco et al. 1996)
		SF-9 insect cells (Logrieco et al. 1996)
Moniliformin	*F. subglutinans* and *F. proliferatum* (Chelkowski et al. 1990, Marasas et al. 1984)	chicks (Kreik et al. 1977)
		ducklings (Kreik et al. 1977)
		rats/mice (Kreik et al. 1977)

Prevalence

F. verticillioides is the most common of the three *Fusarium* species in the tropical and subtropical regions of the world (Logrieco et al. 1993). It is also the dominant *Fusarium* species on maize in the central and southern areas of the United States and southern Europe (Bottalico 1998, Leslie et al. 1990). However, as latitude increases, a shift in dominance occurs so that in the northern United States (Abbas et al. 1988, Munkvold and Stahr 1994, Park et al. 1996), Canada (Vigier et al. 1997), and northern Europe (Bottalico 1998), *F. subglutinans* is more frequently recovered from stalks and kernels than *F. verticillioides*.

Even in South America where *F. verticillioides* is considered the dominant *Fusarium* species (Chulze et al. 1996), *F. subglutinans* is more common in the cooler areas of Peru (Logrieco et al. 1993).

Sources of Inoculum

F. verticillioides, *F. subglutinans*, and *F. proliferatum* mycelia survive in maize residues (Cotten and Munkvold 1998) and serve as sources of inoculum for the following season. Overwintered mycelia can act as inoculum by directly infecting plants or by producing spores that will germinate to infect plants (Nyvall and Kommedahl 1970). Spores are carried through the air by wind and rain (Ooka and Kommedahl 1977) and are introduced to the plants by silk channels, natural openings, and wounds created by insects, animals, and birds. Insects such as *Ostrinia nubilalis* (European corn borer) also can act as vectors by carrying spores on their bodies and introducing them to the kernels (Sobek and Munkvold 1999, Dowd 1998).

Many studies have also shown that infected seed is another source inoculum for *F. verticillioides* (usually reported as *F. moniliforme*). Foley (1962) found *F. moniliforme* survives in the pedicel and abscission layers of the seed. Several years later Sumner (1968) determined that the hyphae grow out of the pedicel and abscission layers of the seed and into the newly developing plant. He further discovered that removal of the pedicels and abscission layers from seeds in a lot with 53% infection could reduce infection to 5% of seeds (Sumner 1968). When infected seeds were allowed to imbibe water for 40 hours, the number of infected embryos and endosperms increased from 2 to 24% (Sumner 1968). This indicated that the beginning of the germination process, the imbibing of water, allows the

fungus to grow from the pedicel into the embryo of the seed. The combined research of Foley (1962) and Sumner (1968) showed that the hyphae of *F. moniliforme* that infected seeds could grow into the roots, mesocotyls, and stems of the new plants causing systemic infection from seed transmission.

During this time period Kucharek and Kommedahl (1966) also conducted a study to determine whether *F. moniliforme* was capable of seed transmission and systemic infection. For this study, seeds from lots with high and low incidences of *F. moniliforme* infection were planted and the plants were sampled. The results of the sampling indicated that the infection of the plants was not significantly different between seed lots (Kucharek and Kommedahl 1966). Kucharek and Kommedahl disagreed with Foley and Sumner about seed transmission and systemic infection caused by *F. moniliforme.*

More recent studies of seed transmission have confirmed Foley and Sumner's conclusion that seed transmission and systemic infection by *F. verticillioides* is an important source of root and stalk infection (Table 3). Kedera et al. (1992), Cotten (1996), Munkvold and Carlton (1997), and Munkvold et al. (1997) inoculated seeds in the lab with different strains of *F. verticillioides,* planted the seeds in the field, and sampled the resulting plants at physiological maturity. All four studies produced results that confirmed Foley and Sumner's research and further suggested seed-to-kernel transmission by the fungus; however, the results of the studies varied in their frequency of seed transmission and systemic infection. The studies differed in fungal strains used, inoculation methods, maize genotypes, and/or environmental conditions.

Table 3. Recent research on seed transmission and systemic infection by *F. verticillioides.* Seeds were inoculated with different strains of the fungus and the plants were sampled at physiological maturity. The values represent the percentage of the plant tissues from which the inoculant strain was recovered.

Researcher	Strain	3rd node	6th node	% ears
Kedera et al. (1992)	498	56	47	15
	501	48	29	7
Cotten (1996)	FM80	2	5	16
	FM42	15	5	15
Munkvold and Carlton	115	0	5	28
(1997)	EA-1	*	*	60-78
Munkvold et al. (1997)	EA-1	*	*	10

* = tissues were not sampled

Markers: Vegetative Compatibility, GUS, and GFP

Fusarium species are ubiquitous and many times the strains used in an experiment are indistinguishable from those that already exist in the environment. This problem has made it necessary to use methods to "mark" strains. Vegetative compatibility, β-glucuronidase (GUS), and green fluorescent protein (GFP) are a few of the approaches used to mark fungal strains in order to study plant-pathogen interactions. All three methods have previously been used to study *Fusarium* species (Munkvold and Carlton 1997, Munkvold et al. 1997, Oren et al. 2001, Yates et al. 1999). These tools have facilitated detection and identification of different *Fusarium* strains *in vitro* and *in planta.*

Vegetative compatibility can be utilized as a marker because clones of a strain are self-compatible. Vegetative compatibility is based on the ability of fungal strains to produce heterokaryons. Heterokaryon production between strains is controlled by vegetative incompatibility (*vic*) loci (Correll et al. 1987). Strains of the same species that successfully produce heterokaryons are placed in vegetative compatibility groups (VCGs) (Leslie 1993). Fungi that are incompatible cannot produce heterokaryons and may act antagonistically

toward each other (Leslie 1993). Because of the high frequency of vegetative incompatibility in *F. verticillioides*, introduced strains are rarely compatible with endemic strains, a characteristic that facilitates the use of vegetative compatibility as a marker.

β-glucuronidase (GUS) production has also been used as a marker to identify strains of filamentous fungi (Freeman et al. 1999, Monke and Schafer 1993) including *F. verticillioides* (Yates et al. 1999). *GusA* is a gene from the bacterium *Escherichia coli* that encodes for GUS production. Fungal strains are transformed with this gene so the enzyme is generated in the cytoplasm of their mycelium (Jefferson 1989). GUS activity can then be detected by the addition of substrates such as X-gluc (5-bromo-4-chloro-3-indoyl glucuronide) (Jefferson 1989, Monke and Schafer 1993). A positive reaction between the enzyme and the substrate makes the mycelium blue in color (Monke and Schafer 1993). False negatives occur when a substrate is not absorbed into the mycelium or the substrate leaks back out of the tissue (Sheen et al. 1995).

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Cubitt et al. 1995) has recently been used to transform filamentous fungi (Maor et al. 1998). The protein is contained in the cytoplasm of fungal mycelia (Gerdes and Kaether 1996) and can be detected when the protein is excited at 395 nm (Cubitt et. al 1995, Quaedvlieg et al. 1998). The result of excitation is a bright, green fluorescence at 598 nm (Cubitt et al. 1995, Quaedvlieg et al. 1998). The gene encoding GFP can be used alone or together with a gene that confers resistance to the antibiotic hygromycin B to provide two methods for screening for transformants. The GFP gene has been used to successfully transform *F. verticillioides*. The transformant was effectively used to study the development of the fungus in maize plants

in planta (Oren et al. 2001). Other types of fluorescent proteins also are currently in use as markers for plant pathogenic fungi (Czymmek et al. 2002).

Research Objectives

Colonization of plant tissues by *Fusarium* species may result in disease or endophytic, symptomless infection (Bacon and Hinton 1996, Yates et al. 1997). Symptomless infection is a concern because mycotoxins are still produced in the affected tissues. Therefore, it is important to understand all of the inoculum sources and pathways of infection for *F. verticillioides*, *F. subglutinans*, and *F. proliferatum*. The objectives for this study are:

1. Determine if *F. subglutinans* can be seed transmitted and cause systemic infection in maize.
2. Determine the effects of temperature on the ability of *F. verticillioides* to be seed transmitted and cause systemic infection in maize.

Thesis Organization

This thesis is organized into four chapters. Chapter one is the general introduction. Chapter two is a paper to be submitted to Phytopathology, entitled "Seed transmission and systemic infection by *Fusarium subglutinans* in maize." Chapter three is a paper to be submitted to Plant Disease, entitled "Seed transmission of *Fusarium verticillioides* in maize plants grown under three different temperature regimes." Chapter four is the general conclusions of this project.

Literature Cited

1. Abbas, H.K., Mirocha, C.J., Meronuck, R.A., Pokorny, J.D., Gould, S.L., and Kommedahl, T. 1988. Mycotoxins and Fusarium spp. associated with infected ears of corn in Minnesota. Appl. Environ. Microbiol. 48:654-661.
2. Bacon, C.W. and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme.* Bot. 74:1195-1202.
3. Bottalico, A. 1988. *Fusarium* diseases of cereals: Species complex and related mycotoxin profiles in Europe. J. Plant Pathol. 80:85-103.
4. Burgess, L. W., Summerell, B. A., Bullock, S., Gott, K. P., and Backhouse, D. 1994. *Laboratory manual for Fusarium research* 3rd edition. Fusarium Research Laboratory Department of Crop Sciences University of Sydney and Royal Botanic Gardens, Sydney.
5. Chelkowski, J. Ed. 1989. Fusarium: Mycotoxins, taxonomy, and pathogenicity. Topics in secondary metabolism, vol. 2. Department of Plant Pathology, Agricultural University, Amersterdam.
6. Chelkowski, J., Zawadzki, M., Zajkowski, P., Logrieco, A., and Bottilico, A. 1990. Moniliformin production by *Fusarium* speices. Mycotoxin Research 6:41-45.
7. Chulze, S.N., Ramirez, M.L., Farnochi, M.C., Pascale, M., Visconti, A., and March, G. 1996. *Fusarium* and fumonisin occurrence in Argentinian corn at different maturity stages. J. Agric. Food Chem. 44:2797-2801.
8. Correll, J.C., Klittich, C.J.R, and Leslie, J.F. 1987. Nitrate nonutilizing mutants of *Fusarium moniliforme*: effects on plants and animals. Science 179:1324-1326.
9. Cotten, T.K. 1996. Survival and seed transmission of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium subglutinans* in maize. M.S. Thesis. Iowa State University. 75 pp.
10. Cotten, T.K. and Munkvold, G.P. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. Phytopathology 88:550-555.
11. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. 1995. Understanding, improving and using green fluorescent proteins. Trends in Biochem. Sci. 20:448-455.
12. Czymmek, K.J., Bourett, T.M., Sweigard, J.A., Carroll, A., and Howard, R.J. 2002. Utility of cytoplasmic fluorescent proteins for live-cell imaging of *Magnapothe grisea* in planta. Mycologia 94(2):280-289.
13. Di Paola, , R., Nena, S., Fornelli, F., Moretti, A., Logrieco, A., Caiaffa, M,F,. Bottalico, A., Tursi, A., and Machia, L. 1994. Cytotoxicity of beauvericin on human B-lymphocyte cell lines. In ICACI XV EAACI 94, Stockholm June 26-July 1. (In press).
14. Dowd, P.F. 1988. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with zenobiotics in *Heliothis zea* (F.) and *Spodoptera frigiperda* (J. E. Smith). Pestic. Biochem. Physiol. 32:123-134.
15. Foley, D.C. 1962. Systemic infection of corn by *Fusarium moniliforme.* Phytopathology 52:870-873.
16. Foley, D.C. 1969. Stalk deterioration of plants and sysceptible to corn stalk rot. Phytopathology 59:620-626.

17. Freeman, S., Maimon, M., and Pinkas, Y. 1999. Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. Phytopathology 89:456-461.
18. Futrell, M.C. and Kilgore, M. 1969. Poor stands of corn and reduction of root growth caused by *Fusarium moniliforme*. Plant Disease Reptr. 53:213-215.
19. Gelderblom, W.C.A., Kriek, N.P.J., Marasas, W.F.O., and Thiel, P.G. 1991. Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B_1, in rats. Carcinogenesis 12: 1247-1251.
20. Gerdes, H. H. and Kaether, C. 1996. Green fluorescent protein: applications in cell biology. FEBS Lett. 389:44-47.
21. Grove, J.F. and Pople, M. 1980. The insecticidal activity of beauvericin and the enniatin complex. Mycopathologia 115:185-189.
22. Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E., and Cole, J.R., Jr. 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. J. Vet. Diagn. Invest. 2:217-221.
23. Jefferson, R.A. 1989. The GUS reporter gene system. Nature (London) 342:837-838.
24. Kabeere, F., Hampton, J.G., and Hill, M.J. 1997. Transmission of *Fusarium graminearum* (Scwabe) from maize seeds to seedlings. Seed Sci. & Technol. 25:245-252.
25. Kedera, C.J., Leslie, J.F., and Claflin, L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. (Abstr.) Phytopathology 82:1138.
26. Kellerman, T.S., Marasas, W.F.O., Thiel, P.G., Gelderblom, W.C.A., Cawood, M.E., and Coetzer, J.A.W. 1990. Leukoencephalomalacia in two horses induced by oral dosing of fumonisins B_1. Onderstepoort J. Vet. Res. 57:269-275.
27. Kingsland, G.C. and Wernham, C.C. 1962. Etiology of stalk rots of corn in Pennsylvania. Phytopathology 52:519-523.
28. Klaasen, J.A. and Nelson, P.E. 1996. Identification of mating population, Gibberella nygamai sp. nov., within the Fusarium nygamai anamorph. Mycologia 88:965-969.
29. Kommedahl, T., Windels, C.E., and Stucker, R.E. 1979. Occurrence of Fusarium species in roots and stalks of symptomless corn plants during the growing season. Phytopathology 69:961-966.
30. Kriek, N.P.J., Marasas, W.F.O., Steyn, P.S., van Rensberg, S.J., Steyn, M. 1977. Toxicity of monliformin-producing strain of *Fusarium moniliforme* var. *subglutinans* isolated from maize. Food Cosmet Toxicol. 15: 579-87.
31. Kucharek, T.A. and Kommedahl, T. 1966. Kernel infection and corn stalk rot caused by *Fusarium moniliforme*. Phytopathology 56:983-984.
32. Kuhlman, E.G. 1982. Varieties of *Gibberella fujikuroi* with anamorphs in Fusarium section *Liseola*. Mycologia 74:759-768.
33. Leslie, J.F. 1991. Mating populations in *Gibberella fujikuroi* (*Fusarium* Section *Liseola*). Phytopathology 81:2058-1060.
34. Leslie, J.F. 1993. Fungal vegetative compatibility. Annu. Rev. Phytopathol. 31:127-150.
35. Leslie, J.F. 1995. *Gibberella fujikuroi*: Avaliable populations and variable traits. Can. J. Bot. 73:S282-S291.

36. Leslie, J.F., Pearson, C.A.S., Nelson, P.E., and Toussoun, T.A. 1990. Fusarium spp. from corn, sorghum, and soybean fields in the central and eastern United States. Phytopathology 80(4):343-350.
37. Leslie, J.F., Plattner, R.D., Desjardins, A.E., and Klittich, C.J.R. 1992. Fumonisin B_1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section Liseola). Phytopathology 82:121-130.
38. Logrieco, A., Moretti, A., Altomare, C., Bottalico, A., and Carbonell Torres, E. 1993. Occurrence and toxicity of *Fusarium subglutinans* from Peruvian maize. Mycopathologia 122:185-190.
39. Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A. Caiaffa, M.F., Randazzo, G., Bottalico, A., and Macchia, L. 1996. Fusaproliferin production by *Fusarium subglutinans* and its toxicity to Artemia salina, SF-9 insect cells, and IARC/LCL 171 human B Lymphocytes. Appl. Environ. Microbiol. 62:3378-3384.
40. Marasas, W.F.O., Miller, J.D., Riley, R.T., and Visconti, A. 2000. Fumonisin B_1. Environmental Health Criteria 219:1-149.
41. Marasas, W.F.O., Nelson, P.E., and Toussoun, T.A. 1984. Toxigenic *Fusarium* species: identity and mycotoxicology. Penn State Univ. Press, State College, PA. pp. 250.
42. Maor, R., Puyesky, M., Horwitz, B.A., and Sharon, A. 1998. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Mycol. Res. 102:491-496.
43. Monke, E. and Schafer, W. 1993. Transient and stable gene expression in the fungal maize pathogen *Cochliobolus heterostrophus* after transformation with the β-glucuronidase (GUS) gene. Mel. Gen. Genet. 241:73-80.
44. Munkvold, G.P. and Carlton, W.M. 1997. Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds. Plant Dis. 81:211-216.
45. Munkvold, G.P. and Leslie, J.F. 1999. Fusarium root rot diseases. Pp. 13 in Compendium of Corn Diseases, Third Edition. D.G. White, Ed. APS Press, St. Paul.
46. Munkvold, G.P., McGee, D.C., and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. Phytopathology 87:209-217.
47. Munkvold, G.P. and Stahr, H.M. 1994. Ear rots and mycotoxins in Iowa corn. (Abstr.) Phytopathology 84:1064.
48. Munkvold, G.P., Stahr, H.M., Logrieco, A., Moretti, A., and Ritieni, A. 1998. Occurrence of fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. Appl. Environ. Microbiol. 64:3923-3926.
49. Nelson, P.E. 1991. History of *Fusarium* systematics. Phytopathology 81(9):1045-1048.
50. Nelson, P.E., Desjardins, A.E., and Plattner, R.D. 1993. Fumonisins, mycotoxins produced by Fusarium species: Biology, chemistry, and significance. Annu. Rev. Phytopathol. 31:233-252.
51. Nelson, P.E., Toussoun, T.A., and Marasas, W.F.O. 1983. *Fusarium species, an illustrated manual for identification*. The Pennsylvania State University Press, University Park and London.

52. Nirenberg, H.I. 1976. Untersuchugen uber die morphologische Differenzierung in der *Fusarium*-Sektion Liseola. Mitt. Biol. Bundesanst. Land-Forstwirtsch. Berlin-Dahlem 169:1-117.
53. Nyvall, R.F. and Kommedahl, T. 1970. Saprophytism and survival of *Fusarium moniliforme* in corn stalks. Phytopathology 60:1233-1235.
54. O'Donnell, K. 1996. Progress towards a phylogenetic classification of *Fusarium*. Sydowia 48(1):57-70.
55. O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. 1998. Molecular systematics and phylogeography of the Gibberella fujikuroi species complex. Mycologia 90(3):465-493.
56. Ojcius, D.M., Zychlinsky, A., Zheng, L.M., and Young, J.D.-E. 1991. Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. Experimental Cell Research 197:43-49.
57. Ooka, J.J. and Kommedahl, T. 1977. Wind and rain dispersal of *Fusarium moniliforme* in corn fields. Phytopathology 67:1023-1026.
58. Oren, L., Ezrati, S., and Sharon, A. 2001. Characterization of *Fusarium*-corn interaction using GFP-transgenic isolate. MPMI Poster Session.
59. Park, J.J., Smalley, E.B., and Chu, F.S. 1996. Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. Appl. Env. Microbiol. 62:1642-1648.
60. Payne, G.A. 1999. Fusarium kernel and ear rot. Pp. 45-46 in Compendium of Corn Diseases Third Edition. D.G. White, Ed. APS Press, St. Paul.
61. Quaedvlieg, N.E.M., Schlaman, H.R.M., Admiraal, P.C., Wijting, S.E., Stougaard, J., and Spaink, H.P. 1998. Fusions between green fluorescent protein and β–glucuronidase as a sensitive and vital bifunctional reporters in plants. Plant Mol. Biol. 37:715-727.
62. Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., and van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. Phytopathology 82:353-357.
63. Ritieni, A., Maria, S.M., Randazzo, G., Logrieco, A., Moretti, A., Peluso, G., Ferracane, R., and Fogliano, V. 1997. Teratogenic effects of fusaproliferin on chicken embryos. J. Agric. Food Chem. 46:3039-3043.
64. Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D.W. 1995. Green-fluorescent protein as a new vital marker in plant cells. Plant J. 8:777-784.
65. Smith, D.R. and White, D.G. 1988. Diseases of corn pp. 687-749, in Corn and Corn Improvement. Sprague, G.F. and Dudley, J.W., eds. American Society of Agronomy, Inc., Crop science Society of America, Inc., and Soil Science Society of America, Inc., Madison.
66. Sobek, E.A. and Munkvold, G.P. 1999. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. J. Econ. Entol. 92(3):503-509.
67. Sumner, D.R. 1968. Ecology of corn stalk rot in Nebraska. Phytopathology 58:755-760.
68. Vigier, B., Reid, L.M., Seifert, K.A., Stewart, D.W., and Hamilton, R.I. 1997. Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario. Can. J. Plant Pathol. 19:60-65.
69. White, D.G. 1999. Fusarium stalk rot. Pp. 41-42 in Compendium of Corn Diseases Third Edition. D.G. White, Ed. APS Press, St. Paul.

70. Yates, I.E., Bacon, C.W., and Hinton, D.M. 1997. Effects of endophytic infection by *Fusarium moniliforme* on corn growth and cellular morphology. Plant Dis. 81:723-728.
71. Yates, I.E., Hiett, K.L., Kapczynski, D.R., Smart, W., Glenn, A.E., Hinton, D.M., Bacon, C.W., Meinersmann, R., Liu, S., and Jaworski, A.J. 1999. GUS tranformation of the maize fungal endophyte *Fusarium moniliforme*. Mycol. Res. 103:129-136.

CHAPTER TWO

SEED TRANSMISSION AND SYSTEMIC INFECTION BY *FUSARIUM SUBGLUTINANS* IN MAIZE

A paper to be submitted to Phytopathology

Amy L. Wilke, Graduate Research Assistant, Charlotte R. Bronson, Professor, and Gary P. Munkvold, Associate Professor

Abstract

Fusarium verticillioides, *F. subglutinans*, and *F. proliferatum* are among the most common fungal pathogens of maize causing root rot, stalk rot, ear rot, seedling blight, and mycotoxin contamination of grain. All three fungi are commonly seedborne, but seed transmission and systemic infection of maize plants has been documented only for *F. verticillioides*. The objective of this study was to determine whether *F. subglutinans* can be seed transmitted and cause a symptomless, systemic infection in maize. *F. subglutinans* strain ITEM2284 was transformed with a gene for green fluorescent protein (GFP). Two greenhouse experiments were conducted in which seeds were inoculated with the transformed strain, planted, and grown to maturity. The roots, mesocotyls, stalks, ears, and kernels of the resulting plants were sampled at growth stages V2, VT, R4, and R6. Sampled tissues were surface disinfested and placed on a semi-selective medium. The identity of the fungal colonies was confirmed by GFP expression. The GFP-expressing strain was recovered from ≥ 87% of the root, mesocotyl, and stem tissues at V2 (seedling stage). At VT (tasseling) and R4 (mid-kernel development), the strain was found in symptomless stalk tissues of the plants up to the ninth internode. At R6 (maturity), the marked strain was found

throughout the stem up to the ninth internode in experiment 1 but not in the ear tissues of the plants. In experiment 2, the strain was found throughout the stalk up to the sixteenth internode and it was also recovered from the ear tissues. The GFP-expressing strain was recovered from the ear shanks of 26% of the inoculated plants, cobs of 20% of the inoculated plants, and kernels of 22% of the inoculated plants; it was recovered from 6% of the 687 kernels sampled. These results demonstrated that *F. subglutinans* can be seed transmitted at a high frequency in maize, and that this species can cause a symptomless, systemic infection of maize plants.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon), *F. subglutinans* (Wollenw & Reinking) Nelson, Toussoun, and Marasas, and *F. proliferatum* (Matsushima) Nirenberg are morphologically similar species and they are among the most common *Fusarium* species infecting maize in most areas of the world. The teleomorph of all three species has been reported as *Gibberella fujikuroi* (Sawada) Wollenw. (21) but they are reproductively distinct (25). *F. verticillioides* (usually reported as *F. moniliforme* Sheld.) has long been considered the predominant species. As a result, *F. subglutinans* and *F. proliferatum* have received limited attention as maize pathogens. *F. verticillioides* is the most common of the three species in the tropical and subtropical regions of the world (28). It is also the dominant *Fusarium* species on maize in the central and southern areas of the United States and southern Europe (5, 26). However, as latitude increases, a shift in dominance occurs so that in the northern United States (1, 34, 46), Canada (56), and northern Europe (5), *F. subglutinans* is more frequently recovered from stalks and kernels than *F.*

verticillioides. Even in South America where *F. verticillioides* is considered the dominant *Fusarium* species (7), *F. subglutinans* is more common in the cooler areas of Peru (28).

These three fungi cause identical diseases of maize (*Zea mays* L.) including root rot, stalk rot, ear rot, and seedling blight. These species also generate toxic secondary metabolites (mycotoxins) that can be produced in symptomatic and symptomless plant tissues (32, 33). When contaminated plant tissues are consumed by animals or humans the mycotoxins within the tissues can cause a variety of health problems.

F. subglutinans produces a variety of mycotoxins including moniliformin (6), beauvericin (28, 37), fusaric acid (13), and fusaproliferin (33, 37). Moniliformin is toxic to mice, rats, ducklings, chicks, and pigs (17, 24). Beauvericin has been found to have toxic effects on brine shrimp (28), human cells (12), insects (16), and murine cells (43). Fusaric acid has been shown to effect insects (13), mice, and swine. Fusaproliferin is toxic to brine shrimp (29), chicken embryos (49), IARC/LCL 171 human B lymphocytes, and SF-9 insect cells (29).

F. verticillioides, *F. subglutinans*, and *F. proliferatum* mycelia survive in maize residues (9) and in seed. Overwintered mycelia can infect plants directly (41) or by produce spores that are dispersed by wind and rain (44) and insect vectors (13, 52).

Most reports on seedborne *Fusarium* have focused on *F. verticillioides* (usually reported as *F. moniliforme*). *F. verticillioides* can be seed transmitted and can be found in symptomless tissues throughout the plant. In 1962, Foley found that *F. moniliforme* survives in the pedicel and abscission layers of the seed. Foley (14) and Sumner (53) determined that hyphae grow out of seeds and into the roots, mesocotyls, and stalks of the new plants. Although Foley reported only on *F. moniliforme*, he did not distinguish between isolates that

did or did not produce microconidia in chains. This suggests that he may have detected *F. subglutinans* as well as *F. verticillioides* (or *F. proliferatum*) in symptomless tissues.

Foley (14) and Sumner (53) used the term systemic infection to describe the symptomless colonization of the plants by *F. moniliforme* because they believed the fungus moved from the roots throughout the whole plant. Many years later Bacon and Hinton (2) described this type of infection as endophytic because they found no evidence of movement of the fungus through the vascular tissues and colonization was only in the intercellular spaces, similar to the relationship between forage grasses and their fungal endophytes.

Kucharek and Kommedahl (20) disagreed with this theory of seed transmission and systemic infection. They planted seeds from lots with high and low incidences of *F. moniliforme* Sheld. emend. Snyd. & Hans.-infection and found that infection of the resulting plants was not affected by the level of seed infection.

Recent studies have confirmed Foley and Sumner's conclusions that seed transmission and systemic infection from *F. verticillioides*-infected seeds is a source of root and stalk infection (34, 35). In addition recent research has demonstrated seed-to-kernel transmission of *F. verticillioides* from infected seed through the plant to developing kernels. Kedera et al. (18) conducted an experiment in which seeds were inoculated in the laboratory with four different strains of *F. verticillioides* and planted in the field. At physiological maturity, the crowns, stalk tissues (nodes), and ear tissues of the resulting plants were sampled and placed on a semi-selective medium to re-isolate the strains (strains were identified using vegetative compatibility). The inoculated strains were recovered from 65% of the crowns, 34 to 54% of the nodes, 10% of the cobs, and 8% of the seeds.

Another study conducted by Desjardins and Plattner (11) supports the occurrence of systemic infection and further suggests that this may contribute to mycotoxin contamination in grain. A strain of *F. verticillioides* that produced only fumonisin B_2 (FB_2) but not FB_1 or FB_3, was applied to seeds at planting. At physiological maturity, fungi were isolated from the kernels and their fumonisin production was characterized. From the plants that were grown from seeds inoculated with the FB_2-producing strain, 47 to 78% of the strains recovered from the kernels produced only FB_2. From these results it was concluded that the FB_2 producing strain systemically infected the plants and produced FB_2 in the kernels.

Other *Fusarium* species have been detected in symtomless maize tissues (19) but they have not been reported as "systemic" or "endophytic." Similar studies have not been conducted with *F. subglutinans* even though it is the dominant *Fusarium* species in maize kernels and stalks in certain areas of the world. We hypothesize that because *F. subglutinans* and *F. verticillioides* are morphologically similar *F. subglutinans* also could be seed transmitted and systemically infect maize plants. Because of reductions in yield and quality caused by *F. subglutinans* and the toxic effects of its secondary metabolites, it is important to understand inoculum sources and pathways for infection of maize by this fungus.

A marker such as green fluorescent protein (GFP) is valuable in studies with *Fusarium* species because *Fusarium* species are ubiquitous and background infection makes it difficult to identify inoculant strains. GFP from the jellyfish *Aequorea victoria* (10) has recently been used to transform filamentous fungi (31). The protein is contained in the cytoplasm of fungal mycelium (15) and can be detected when the protein is excited at 395 nm (10, 47). The result of excitation is a bright, green fluorescence at 598nm (10, 55). The gene encoding GFP can be used alone or together with the *hph* gene that confers a resistance

to the antibiotic hygromycin B, providing two methods for screening for transformants. The GFP gene has been used to successfully transform *F. verticillioides*. The transformant was used to study the development of the fungus in maize plants *in planta* (45).

In this study, we used a GFP transformant of *F. subglutinans* strain ITEM2284 (TXW-99) to determine whether this species of *Fusarium* can be seed transmitted and cause symptomless, systemic infection in maize.

Materials and Methods

A *Fusarium* strain was isolated from Iowa maize kernels in 1996 (37) and identified as *F. subglutinans* based on morphological characteristics (40). The strain was designated ITEM2284. The strain was confirmed as belonging to mating population E of *Gibberella fujikuroi* (25) based on sexual compatibility with established tester strains (37).

Transformation. *F. subglutinans* strain ITEM2284 was transformed with genes for green fluorescent protein (GFP) and hygromycin B resistance using plasmid pIGPAPA, generously provided by B. Gillian Turgeon (Cornell University, NY).

Plasmid pIGPAPA contains the gene sGFP attached to a truncated (and therefore constitutive) isocitrate lyase promoter from *Neurospora crassa*. It also includes a hygromycin B phosphotransferase gene (*hygB*) for selection in fungi and an ampicillin resistance gene for selection in *Escherichia coli*. The plasmid was purified using QIAfilter Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) and was linearized with *Bam*HI.

Protocols for preparation of protoplasts were adapted from methods described for *Cochliobolus heterostrophus* by Turgeon et al. (54). Strain ITEM2284 was grown on complete medium with xylose (CMX) (23) for 7 to 10 days. A conidial suspension (1.09×10^4 conidia/ml) was prepared in liquid complete medium (CM) (23) and incubated at 30°C

for 20 hours on a rotary shaker at 150 rpm. The resulting mycelial mass was pelleted and then re-suspended in 0.7 NaCl solution. The mycelia were then incubated at 30°C for 1.5 hours in enzyme osmoticum (0.1 mg /ml chitinase (Sigma, St. Louis, MO), 7.5 mg driselase/ml (Sigma, St. Louis, MO), and 7.5 mg Novozyme 234/ml (InterSpex Products Inc., Foster City, CA)) (30) to digest the cell walls. Protoplasts were harvested by filtering the enzyme-osmoticum through two layers of cheesecloth and one layer of Nitex (pore size 20 μm, Tetko Inc., Elenstord, N.Y.). The filtrate was centrifuged for 15 min at 1600 × g and then decanted. The pellet was washed by adding 10 ml of cold STC (1.2 M sorbitol, 10mM Tris (pH 7.5), and 50 mM $CaCl_2$) and centrifuged for 15 min. The supernatant was decanted and the pellet was then re-suspended in 1 ml of STC.

Protocols for transformation were the same as described for *C. heterostrophus* by Turgeon et al. (54) except for the use of regeneration medium in place of molten acetamide medium. Thirty μg of linearized plasmid DNA were added to a tube with approximately 2.8 × 10^7 protoplasts in 100 μl of STC. Three additions of polyethylene glycol (PEG) solution were made every 10 minutes in aliquots of 200 μl, 200 μl, and 800 μl. During these incubation periods the tube was rolled to mix its contents. One ml of STC was added to dilute the PEG solution. The contents of the tube were then added to regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose, and 2% agar) and incubated at 30°C for 24 hours to allow protoplasts to regenerate walls and grow into colonies of mycelia. The plates were overlayed with 20 ml of 1% water agar with 100 μg/ml hygromycin B (Roche, Indianapolis, IN). Colonies that were transformed were resistant to hygromycin B and therefore grew through the overlay in 4 to 5 days.

Plugs from the resulting colonies were transferred to petri dishes of CMX with hygromycin B. Colonies that were not inhibited by the antibiotic were checked for GFP expression using a fluorescence microscope. Eighteen colonies were confirmed as transformants based on their resistance to hygromycin B and their ability to fluoresce. The transformants were then placed on silica gel for storage at 4°C (55).

Transformant characterization. Three of the transformants were tested to confirm that the gene for GFP was mitotically stable. Two mitotic stability tests were conducted with GFP transformants of *F. subglutinans* strain ITEM2284: TXW-4, TXW-36, and TXW-99. The transformants were chosen based on their ability to fluoresce and rapid radial growth on CMX that was similar to the wild type (ITEM2284) growth rate.

In the first test, sequential transfers were conducted to determine if gene expression was stable. The test was started by placing a single spore of the transformant on the outside edge of a petri dish containing CMX. For each transformant, three replicates were made. The petri dishes were incubated at 24°C under fluorescent lighting. When the colony covered the agar surface, a plug of mycelium was taken from the growing margin and transferred to the edge of a new plate of CMX. Four cycles of growth and transfer were done in this manner. Mycelium from the original petri dishes and the sequential transfers was checked for GFP expression using a fluorescence microscope and the results were recorded as positive or negative.

In a second test, stability of GFP expression through sporulation was tested. Spore suspensions (100 spores/ml) of transformants TXW-4, TXW-36, and TXW-99 were prepared and 1 ml of spore suspension was spread on each of 10 petri dishes of CM and sorbose (23) for each transformant. Sorbose was used to restrict colony size. The dishes were incubated

at 23 to 25°C under fluorescent lights for several days until the resulting colonies were approximately 1 to 3 mm in diameter. Each colony was checked for GFP expression using a fluorescence microscope. The number of colonies with GFP expression was recorded.

An experiment was conducted to determine if transformation had affected the growth of the fungus in liquid culture. Spore suspensions ($1.1x10^6$ spores/ml) of TXW-99 (transformant) and ITEM2284 (wild type) were made. In 125-ml Erlenmeyer flasks, 1 ml of spore suspension was added to 29 ml of liquid CMX. For each strain, nine flasks of medium were inoculated. The flasks were placed on a rotary shaker at 100-125 rpm at 23 to 25°C. After 2 weeks, the contents of each flask were vacuum filtered using pre-weighed filter papers. After filtration the filter papers containing mycelium were placed in petri dishes left partly open. All of the petri dishes were placed in a 26°C incubator to dry for 1 week. The filter papers with mycelia were then weighed and the dry weight of the mycelial mass was calculated.

A test also was conducted to determine if transformation had affected the ability of TXW-99 to colonize maize stalk tissues. Thirty seeds of Cargill hybrid 1077 (Cargill Inc., Minneapolis, MN) were surface disinfested (2 min in 0.5% NaOCl and 0.5 min in sterile distilled water), planted in pots filled with pasteurized soil (1 part peat: 2 parts soil: 1 part perlite), and placed in the greenhouse. At tasseling (VT) the plants were inoculated with either TXW-99 or strain ITEM2284 (wild type). Spore suspensions ($1x10^{-6}$ spores/ml) of each fungus were made from 7 to 14-day-old cultures 24 hours before the inoculations and kept at 4°C. Corn plants were injected by syringe just above the second leaf node with 0.5 ml of spore suspension. Ten plants were inoculated with TXW-99 and five plants with strain ITEM2284. The stalk of each plant was harvested 46 days after inoculation. The stalks were

cut into 30-cm pieces and disinfested for 3 min in 70% ethanol, 5 min in 0.5% NaOCl, and 2 min in sterile distilled water. Internodes were numbered and the center 2 cm portion of each internode (starting just above the second leaf node) was excised from the stalk, split longitudinally, and placed inner surface down on the medium. Tissues of plants that were inoculated with ITEM2284 were placed on Nash-Snyder medium (Nash and Snyder 1962) without PCNB. The tissues of plants inoculated with TXW-99 were placed on Nash-Snyder medium without PCNB and with 100 µl of hygromycin B per liter of medium. Incorporating hygromycin B made the medium selective for the transformants but preliminary experiments indicated that the combination of this antibiotic with PCNB (an ingredient of Nash-Snyder medium) was too inhibitory. The dishes were incubated at room temperature in the dark for 4 to 5 days to allow fungal mycelium to grow out of the tissues. Colonies were then stored at 4°C until the fungal mycelium could be checked for appropriate morphological characteristics and/or GFP expression.

Greenhouse experiments. Two experiments were conducted in the greenhouse to assess seed transmission of and systemic infection of maize by *F. subglutinans*.

A spore suspension ($1x10^6$ spores/ml) was made from 7 to 14-day-old cultures of TXW-99 grown on CMX and kept at 4°C for 10 hours or less until it was used as inoculum. Seeds of Cargill 1077 were surface disinfested (2 min in 10% bleach and 0.5 min in sterile distilled water), placed on sterile paper towels, and allowed to dry in a laminar flow hood. After the seeds were dry they were placed in 125-ml Erlenmeyer flasks, 15 to 25 seeds per flask, with either 25 to 50 ml of spore suspension or sterile distilled water. The flasks were placed on a rotary shaker at 100 to 125 rpm for 12 hours. The inoculated and non-inoculated seeds were then placed on sterile paper towels and allowed to dry in separate hoods (a

biological safety cabinet and a other laminar-flow). After the seeds had dried they were taken to the greenhouse to be planted.

Prior to planting, a sample of 50 inoculated seeds was tested for infestation and infection by TXW-99. Twenty-five seeds were placed on plates of Nash-Snyder medium without PCNB and with hygromycin B. The other 25 were surface disinfested in 70% ethanol for 3 min, 10% bleach for 5 min, and sterile distilled water for 2 min and placed on the same medium. The plates were then incubated in the dark at 23 to 25°C for 4 to 5 days. Fungal mycelium was then checked for GFP expression and the number of seeds yielding TXW-99 was recorded.

In the greenhouse, pots were placed in a single greenhouse space containing three large tables. Different pot sizes were used depending on the growth stage (48) that the plants were to be sampled. Plants that were to be sampled at the seedling stage (V2) were planted in 4" pots, plants sampled at tasseling (VT) in 6" pots, and plants sampled at either dough stage (R4) or physiological maturity (R6) were planted in 10" pots. All of the pots were filled with pasteurized soil (1 part vermiculite: 2 parts soil: 1 part peat by volume), double planted (2 seeds per pot) 3 to 5-cm deep, and thinned after emergence to one seedling per pot.

Experiment 1 was set up in a split-plot design with three replicate blocks. Main plots were sampling date and subplots consisted of individual plants grown either from inoculated or non-inoculated seed. For each sampling date, 25 pots of inoculated seeds and 10 pots of non-inoculated seeds were arbitrarily arranged. Therefore, for all three blocks, there was a total of 75 inoculated seeds and 30 non-inoculated seeds planted for each sampling date. In experiment 2, the greenhouse room was again divided into three blocks but for this

experiment a randomized complete block design was used. Pots containing inoculated and non-inoculated seeds were randomized. For each sampling date, 24 pots of inoculated seeds and 8 pots of non-inoculated seeds were placed in a block for a total of 72 inoculated seeds and 24 non-inoculated seeds.

Experiment 1 and experiment 2 were conducted at different times of the year and the growing conditions in the greenhouse varied accordingly. Experiment 1 was conducted over the months of August to December of 2000 and experiment 2 was conducted over the months of April to August of 2001. During experiment 1, the temperatures of the greenhouse ranged from 20°C to 40°C from August to October and during November and December the temperatures ranged from 15°C to 25°C. In experiment 2, the temperatures for the month of April ranged from 15°C to 25°C and ranged from 20°C to 40°C for May to August. Day length in both experiments also varied with the time of the year and no supplemental lighting was used.

Pots in both experiments were carefully hand-watered to prevent splashing between pots. At planting, pots were watered to saturation. After planting, pots were watered to maintain adequate soil moisture. Plants were also fertilized with a water soluble fertilizer (21-5-20, Miracle-Gro Excel, Scotts Company, Marysville, OH) every two weeks starting after growth stage V2.

An Anderson air sampler was used to determine if any spores from TXW-99 had become air-borne in the greenhouse room. The sampler was placed on the center of the middle greenhouse table and run for two 15-min periods. For the first 15 min period the sampler was utilized, it was filled with 6 plates of Nash-Snyder without PCNB and for the second 15 min period it was filled with 6 plates of Nash-Snyder without PCNB and with

hygromycin B. The plates were incubated in the dark at 23 to 25°C for 4 to 5 days. The resulting colonies were counted and checked for GFP expression by using a fluorescence microscope if they were suspected to be *F. subglutinans*, TXW-99. In experiment 1, the sampler was used once before the V2 sampling date. In experiment 2, the sampler was utilized once a week for the duration of the experiment.

The plants that were sampled at the R4 and R6 sampling dates were hand-pollinated. The inoculated plants were detasseled at VT to prevent possible contamination by sporulation of TXW-99 from the tassel (27). Only pollen from the non-inoculated plants was used. In experiment 1, the tassels were bagged immediately after emerging from the whorl. On the day of pollination, when the silks had emerged, the tassels were shaken gently to release pollen. Then the pollen that had been captured in the bags was distributed to the new ear silks. However, bagging the tassels at their emergence apparently reduced the amount of available pollen. This resulted in poor pollination and reduced seed fill. For experiment 2, the tassels were not enclosed in bags until the day pollen was needed. Then the bags were placed on the tassels and the tassels were shaken to release pollen. The pollen collected in the bags was then distributed to the new ear silks. This method allowed the tassels to produce more pollen that resulted in better pollination and seed fill.

Whole plants were sampled at stages V2, VT, R4, and R6. Seedlings (V2) were harvested and then disinfested (3 min in 70% ethanol, 5 min in 10% bleach, and 2 min in sterile distilled water). The mesocotyl and stem (portion of the plant between the mesocotyl and the first leaf collar) were measured. The seed and approximately 10-cm of the primary root (five 2-cm pieces) were then placed on Nash-Snyder medium without PCNB and with hygromycin B. The mesocotyl and stem were then cut longitudinally and the inner surfaces

were placed directly on the medium. Petri dishes were placed in clear, plastic containers and kept in the dark at 23 to 25°C for 4 to 5 days to allow fungal growth from the tissues. The growth was then checked for GFP expression using a fluorescence microscope. The percentage of samples of each tissue type yielding TXW-99 was recorded.

At stage VT (tasseling), plants were measured from the soil surface to the collar of the flag leaf and then harvested (removed from the pots and cut into 30-cm pieces). Plants were surface disinfested as previously described. Five 2-cm pieces of the primary root were placed on Nash-Snyder medium without PCNB and with hygromycin B. The mesocotyl, shank of the ear, and the center 2-cm of each internode were all cut longitudinally and placed with the inner surfaces down on the medium. Internodes were sampled to avoid detection of possible local infections occurring at the nodes. The top 0.5-cm and the bottom 0.5-cm of each ear shoot also were cultured. Petri dishes were placed in clear, plastic containers to prevent dessication and kept in the dark at 23 to 25°C for 4 to 5 days. Fungal growth from the tissues was checked for GFP expression. The percentage of samples of each tissue type yielding TXW-99 was recorded.

At the dough stage (R4) and physiological maturity (R6) the plants were measured from the soil surface to the collar of the flag leaf and then harvested from their pots and cut into 30-cm pieces. The plants were surface disinfested. The primary root, mesocotyl, the internodes, the shank of the ear, and the cob were sampled and cultured for fungal isolation as already described. In addition to these tissues, up to 10 kernels from each ear were sampled and cultured on the same medium. If an ear had less than 10 kernels, then all of the kernels were sampled. On ears with more than 10 kernels, the kernels were selected arbitrarily. Petri dishes were incubated in clear, plastic containers and kept in the dark at 23

to 25°C for 4 to 5 days. The resulting fungal growth was checked for GFP expression. The percentage of samples of each tissue type yielding TXW-99 was recorded.

The data from experiment 1 and experiment 2 were analyzed (using PROC GLM analysis of variance, SAS version 6.12) to determine if the data generated by the two experiments were significantly different for the V2, VT, R4, and R6 sampling dates. Analysis showed that for the two experiments the recovery of the fungus from plant tissues sampled at V2 was significantly different (P=0.0030). At VT and R4, recovery of the fungus was not significantly different (P=0.8867 and P=0.8977, respectively) but the recovery at the R6 sampling date differed between experiments (P=0.0033). The data from experiments 1 and 2 were therefore combined for VT and R4. For R6, the data from experiments 1 and 2 were analyzed separately. The data were analyzed to detect significant differences in the recovery of TXW-99 from tissues of plants grown from inoculated seeds versus plants from non-inoculated seeds.

Results

The mitotic stability results indicated that GFP expression was stable through vegetative growth and sporulation in the transformed *F. subglutinans* strains. In the first experiment, for each transformant tested (TXW-4, TXW-36, and TXW-99), four out of four consecutive transfers for all three replicates maintained GFP expression. In the second experiment, all 1086, 1130, and 828 colonies derived from conidia of TXW-4, TXW-36, and TXW-99 respectively, expressed GFP (Table A.1). From these results we concluded that GFP would be an effective marker for *F. subglutinans* strain ITEM2284.

Transformation did not affect the growth of transformant TXW-99 in liquid culture. There was no significant difference in dry weight between TXW-99 and ITEM2284 (P=0.7067), indicating that the strains grew at comparable rates (Table A.2).

Transformant TXW-99 and the wild type ITEM2284 colonized maize stalk tissues at similar rates. A statistical analysis was conducted to compare the highest internode of each plant from which TXW-99 and ITEM2284 were recovered (Table A.3). There was no significant difference in the extent of colonization by the two strains (P=0.1499). From this we concluded that transformation did not affect the ability of the fungus to colonize maize stalk tissues.

The Anderson air sampler collected spores of TXW-99 during both experiments. In experiment 1, more than 200 fungal spores were captured on the plates from air sampling on conducted just prior to the V2 sampling date. The resulting colonies that had morphological characteristics similar to *Fusarium* species were checked for GFP expression using a fluorescence microscope. Only two colonies found had GFP expression and were identified as TXW-99. In experiment 2, the number of spores that were collected weekly ranged from a few to more than 300. Again colonies that were suspected to be a species of *Fusarium* (based on colony morphology) were checked for GFP expression. Only one spore of TXW-99 was collected during the duration of the experiment. The spore was captured just prior to the R4 sampling date. The majority of the spores collected during the experiments were *Aspergillus* and *Penicillium* species. Airborne movement of TXW-99 was negligible and probably not a source of plant infection.

Our inoculation method provided high levels of both seed infestation and infection. For experiment 1, 25 out of 25 (100%) seeds that were cultured without surface disinfestation

were infested with TXW-99. Twenty out of 25 (80%) seeds that were surface disinfested and cultured were infected with TXW-99. For experiment 2, and 25 out of 25 (100%) were infested and 22 out of 25 seeds (88%) were infected.

Plant height was compared for plants grown from inoculated and non-inoculated seeds for each sampling date (V2, VT, R4, and R6) using a PROC GLM statistical analysis. The analysis was conducted to determine if seed inoculation affected plant growth. The height of the plants at all growth stages was significantly different between experiment 1 and experiment 2. For the V2 sampling date there was an interaction between seed inoculation and experiment (P=0.0012). Seed inoculation did not significantly affect plant height (P=0.4967) in experiment 1 but it did in experiment 2 (P=0.0025). For the V2 sampling date in experiment 2, mean height was 3.84 cm for inoculated plants and 4.60 cm for non-inoculated plants. The interaction between seed inoculation and experiment was not significant for VT, R4, and R6 (P=0.9438, P=0.2200, and P=0.3098, respectively). For these sampling dates, seed inoculation did not affect plant height (P=0.2585, P=0.2840, and P=0.4821).

In both greenhouse experiments, TXW-99 was recovered from the below-ground and above-ground tissues of the plants grown from inoculated seeds. The recovery of TXW-99 from specific tissues of plants grown from inoculated seeds was similar between the experiments for V2, VT, and R4 but not for R6. For the V2 sampling date, the recovery of TXW-99 from the root, mesocotyl, and stem tissues was ≥87% in experiment 1 and ≥99% in experiment 2 and the transformant was not isolated from non-inoculated plants (Fig. 2.1). Recovery of TXW-99 from plant tissues grown from inoculated seeds was significantly different from non-inoculated seeds for all tissues in both experiments $P \leq 0.0002$.

At VT, R4, and R6 for both experiments, systemic infection in the below-ground tissues (internode three and below) was nearly 100% for the plants grown from inoculated seeds. However, recovery of TXW-99 from above-ground tissues declined as the distance between the tissues and the soil surface increased (Figs. 2.2 and 2.3). At VT, TXW-99 was recovered from the below-ground tissues and above-ground tissues up to the sixth internode during experiment 1 and up to the seventh internode in experiment 2. The results of the experiments were combined to determine significant differences between the recovery of TXW-99 from tissues of plants grown from inoculated seeds and non-inoculated seeds. Significant differences were detected for the tissues between and including the root and the fifth internode. At R4, the fungus was recovered from the tissues up to the ninth internode in experiment 1 and up to the eighth in experiment 2. The results of these sampling dates also were combined for analysis. Recovery from inoculated plants was significantly different from non-inoculated plants for tissues from the root to the sixth internode. At R6, the fungus was recovered from the tissues up to the ninth internode in experiment 1 but in experiment 2, TXW-99 moved much further up the stalk (internode 15) (Fig. 2.3). The results from experiment 1 and experiment 2 were analyzed separately. In experiment 1, significant differences between inoculated and non-inoculated treatments were detected in the tissues from the root to the fifth internode, and in experiment 2; significant differences were found for tissues from the root to the twelfth internode. In experiment 2 at R6, TXW-99 was also recovered from the ear tissues of the plants (Fig. 2.4). The fungus was recovered from the ear shanks of 26% of the inoculated plants, cobs of 20% of the inoculated plants, and kernels of 22% of the inoculated plants. Of the 687 kernels sampled, the fungus was recovered from 6%. Recovery of TXW-99 was irregular from tissues grown from non-inoculated seeds and

ranged from 0 to 7% at VT, 0 to 10% at R4, and 0 to 2% at R6. TXW-99 was not recovered from ear tissues in the non-inoculated treatment.

Discussion

We found that *F. subglutinans* strain TXW-99 was seed transmitted and caused symptomless, systemic infection similar to that of *F. verticillioides* (18, 22). Ninety to one hundred percent of plants grown from TXW-99-inoculated seeds were infected even though seed infection was only 80% in experiment 1 and 88% in experiment 2. This suggests that the infection process of *F. subglutinans* can start from either infected seeds or infested seeds, similar to *F. verticillioides*. Infection of plants from seeds infested by *F. verticillioides* has been shown to occur through the epidermis and through cortical wounds produced by adventitious roots (22). A similar process for *F. subglutinans* would explain the high frequency of infection (90 to 100%) in the below-ground plant tissues in our experiments which must have resulted from the high incidence of infestation (100%). Desjardins and Plattner (11) also found that by planting *F. verticillioides*-infested toothpicks near non-inoculated seeds the inoculant strain could be recovered from the kernels of the resulting plants (47 to 78%). This further suggests that seed infection is not necessary for systemic infection to occur but that it can be initiated by the fungus merely residing in the immediate proximity of the seed.

To our knowledge this is the first report of symptomless systemic infection and seed-to-kernel transmission by *F. subglutinans* in maize. At the early growth stage of V2, TXW-99 was recovered from the roots and crowns of the plants as has been reported for *F. verticillioides*. During the later growth stages (VT and R4), TXW-99 moved through the stalks of the plants and could be recovered from tissues below the ninth internode. *F.*

verticillioides also advances from the crown to the rest of the stalk tissues beginning at VT (22). At R6 in experiment 2, TXW-99 was recovered from the entire plant including the ear tissues as Kedera et al. (18) found with *F. verticillioides* at full maturity. In our experiment, TXW-99 was recovered from 39% of the internodes associated with the ear. Kedera et al. (18) recovered *F. verticillioides* from 34% of the seventh nodes. Recovery of the inoculant strains from the kernels was 6% for our experiments and 8% for Kedera et al. (18).

There are two possible explanations for the difference between the results of experiment 1 and experiment 2 at growth stage R6: pollination success and temperature. Pollination causes physiological changes in the plants such as the carbohydrate transport that occurs from the roots to the developing kernels (48). These physiological changes may act as a trigger for the fungus causing it to move further up the stalks and into the ear tissues (22). The poor pollination that occurred in experiment 1 resulted in development of only a few kernels per ear, which may not have been enough to cause the usual physiological changes. This may explain why TXW-99 was not recovered above the ninth internode or in the ear tissues. Pollination in experiment 2 was considerably better and ultimately so was seed fill. In this experiment, TXW-99 was recovered from the all internodes up to the fifteenth internode and from the ear tissues.

It is also possible that differences in temperature may have affected the ability of the fungus to colonize the tissues and thus affected the results of the experiments. The optimal temperature for *F. subglutinans* growth is approximately 25°C (4). Root and stem elongation of maize occurs best at 30°C (3). The temperatures in the greenhouse for experiment 1 ranged from 15°C to 25°C during pollination, seed fill, and maturity and in experiment 2, the temperatures ranged from 20°C to 40°C during the same period. The temperatures were

therefore mostly below the optimal temperature for *F. subglutinans* in experiment 1 and at or above the optimal temperature in experiment 2. It is possible that the cooler temperatures in experiment 1 slowed the growth of the fungus compared to the higher temperatures in experiment 2. This could have contributed to the more extensive plant colonization that occurred in experiment 2.

Contamination of the tissues of non-inoculated plants was negligible (≤10% for any one tissue) and recovery of airborne spores was also very rare. Infection of the tissues of the inoculated plants therefore was considered primarily systemic.

Significant differences in plant height between experiment 1 and experiment 2 were likely due to the varied conditions in the greenhouse. These two experiments were conducted during different seasons so temperature and day length were variable and plant height was significantly different in experiment 1 and experiment 2 for each growth stage. The warmer temperatures in experiment 2 were likely more favorable for rapid plant growth.

The GFP construct used in these experiments was a suitable marker for *F. subglutinans* strain ITEM2284 (transformant TXW-99) and it allowed us to distinguish this strain from other naturally occurring *Fusarium* strains in the greenhouse environment. GFP also proved to be more reliable and convenient than vegetative compatibility which has been used as a marker in previous studies (8, 34, 36). Unlike GFP, which was maintained during our experiments, vegetative compatibility can be lost if auxotrophic mutants become dysfunctional and can not form heterokaryons or revert back to wild-type growth (8). Obtaining the results of an experiment is also faster and easier with GFP than with vegetative compatibility testing, which is labor-intensive.

F. subglutinans strains are capable of seed transmission and systemic infection but it is unclear what role this process may play in mycotoxin accumulation in maize kernels. Desjardins and Plattner (11) found that by applying an FB_2 producing, *F. verticillioides* strain to seeds at planting that more FB_2 producing strains could be recovered from the kernels of the resulting plants. This suggests that for *F. verticillioides* there is a relationship between symptomless, systemic infection and the accumulation of mycotoxins in the kernels. It is reasonable to believe that this kind of relationship also exists for *F. subglutinans* due to the morphological and epidemiological similarities it has to *F. verticillioides*. Further studies similar to Desjardins and Plattner (11) should be conducted to elucidate the relationship between systemic infection of maize by *F. subglutinans* and mycotoxin accumulation in the kernels.

Literature Cited

1. Abbas, H.K., Mirocha, C.J., Meronuck, R.A., Pokorny, J.D., Gould, S.L., and Kommedahl, T. 1988. Mycotoxins and *Fusarium* spp. associated with infected ears of corn in Minnesota. Appl. Environ. Microbiol. 48:654-661.
2. Bacon, C.W. and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme.* Can. J. Bot. 74:1195-1202.
3. Blacklow, W.M. 1972. Influence of temperature on germination and elongation of the radicle and shoot of corn (Zea mays L.). Crop Sci. 12:647-650.
4. Booth, C. 1971. The Genus Fusarium. Commonwealth Mycological Institute, Kew. pp. 237.
5. Bottalico, A. 1998. *Fusarium* diseases of cereals: species complex and related mycotoxin profiles in Europe. J. Plant Pathol. 80:85-103.
6. Chelkowski, J., Zawadzki, M., Zajkowski, P., Logrieco, A., and Bottilico, A. 1990. Moniliformin production by *Fusarium* speices. Mycotoxin Research 6:41-45.
7. Chulze, S.N., Ramirez, M.L., Farnochi, M.C., Pascale, M., Visconti, A., and March, G. 1996. *Fusarium* and fumonisin occurrence in Argentinian corn at different maturity stages. J. Agric. Food Chem. 44:2797-2801.
8. Cotten, T.K. 1996. Survival and seed transmission of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium subglutinans* in maize. M.S. Thesis. Iowa State University. 75 pp.
9. Cotten, T.K. and Munkvold, G.P. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. Phytopathology 88:550-555.
10. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. 1995. Understanding, improving and using green fluorescent proteins. Trends in Biochem. Sci. 20:448-455.
11. Dejardins, A.E., and Plattner, R.D. 2000. Fumonisin B_1 – nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. J. Agric. Food Chem. 48:5773-5780.
12. Di Paola, , R., Nena, S., Fornelli, F., Moretti, A., Logrieco, A., Caiaffa, M,F,. Bottalico, A., Tursi, A., and Machia, L. 1994. Cytotoxicity of beauvericin on human B-lymphocyte cell lines. In ICACI XV EAACI 94, Stockholm June 26-July 1. (In press).
13. Dowd, P.F. 1998. Involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions. Pp. 307-350 in Mycotoxins in Agriculture and Food Safety, K.K. Sinha and D. Bhatnagar, Eds. Marcel Dekker, Inc. NY.
14. Foley, D.C. 1962. Systemic infection of corn by *Fusarium moniliforme.* Phytopathology 52:870-873.
15. Gerdes, H. H. and Kaether, C. 1996. Green fluorescent protein: applications in cell biology. FEBS Lett. 389:44-47.
16. Grove, J.F. and Pople, M. 1980. The insecticidal activity of beauvericin and the enniatin complex. Mycopathologia 115:185-189.

17. Harvey, R.B., Kubena, R.F., Rottinghaus, G.E., Turk, J.R., and Buckley, S.A. 1997. Effects of fumonisin and moniliformin from culture materials to growing swine. Cereal Research Communications 25:415-417.
18. Kedera, C.J., Leslie, J.F., and Claflin, L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. (Abstr.) Phytopathology 82:1138.
19. Kommedahl, T., and Windels, C.E. 1981. Root-, stalk-, and ear-infecting *Fusarium* species on corn in the USA. Ch. 9, pp. 94-104 in *Fusarium*: Diseases, Biology, and Taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., Eds. Pennsylvania State Univ. Press, University Park, PA.
20. Kucharek, T.A. and Kommedahl, T. 1966. Kernel infection and corn stalk rot caused by *Fusarium moniliforme*. Phytopathology 56:983-984.
21. Kulman. E.G. 1982. Varietes of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola*. Mycologia 74(5):759-768.
22. Lawrence, E.B., Nelson, P.E., and Ayers, J.E. 1981. Histopathology of sweet corn seed and plants infected with *Fusarium moniliforme* and *F. oxysporum*. Phytopathology 71:379-386.
23. Leach, J., Lang, B.R., and Yoder, O.C. 1982. Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*. J. Gen. Microbiol. 128:1719-1729.
24. Ledoux, D.R., Bermudez, A.J., Rottinghaus, G.E. 1995. Effects of feeding *Fusarium fujikuroi* culture material, containing known levels of moniliformin, in young broiler chicks. Poultry Science 74:297-305.
25. Leslie, J.F. 1995. *Gibberella fujikuroi*: Avaliable populations and variable traits. Can. J. Bot. 73:S282-S291.
26. Leslie, J.F., Pearson, C.A.S., Nelson, P.E., and Toussoun, T.A. 1990. Fusarium spp. from corn, sorghum, and soybean fields in the central and eastern United States. Phytopathology 80(4):343-350.
27. Logrieco, A. and Bottalico, A. 1987. Presenza di specie di *Fusarium* e relative forme ascofore sulle infiorescenze maschili e sugli stili di Mais. Phytopathology 26:147-150.
28. Logrieco, A., Moretti, A., Altomare, C., Bottalico, A., and Carbonell Torres, E. 1993. Occurrence and toxicity of *Fusarium subglutinans* from Peruvian maize. Mycopathologia 122:185-190.
29. Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A. Caiaffa, M.F., Randazzo, G., Bottalico, A., and Macchia, L. 1996. Fusaproliferin production by *Fusarium subglutinans* and its toxicity to Artemia salina, SF-9 insect cells, and IARC/LCL 171 human B Lymphocytes. Appl. Environ. Microbiol. 62:3378-3384.
30. Lu, S., Lyngholm, L., Yang, G., Bronson, C., and Yoder, O.C. 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated intergration. Proc. Natl. Acad. Sci. 91:12649-12653.
31. Maor, R., Puyesky, M., Horwitz, B.A., and Sharon, A. 1998. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. Mycol. Res. 102:491-496.
32. Marasas, W.F.O., Miller, J.D., Riley, R.T., and Visconti, A. 2000. Fumonisin B_1. Environmental Health Criteria 219:1-149.

33. Marasas, W.F.O., Nelson, P.E., and Toussoun, T.A. 1984. Toxigenic *Fusarium* species: identity and mycotoxicology. Penn State Univ. Press, State College, PA. pp. 250.
34. Munkvold, G.P. and Carlton, W.M. 1997. Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds. Plant Dis. 81:211-216.
35. Munkvold, G.P., McGee, D.C., and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme.* Phytopathology 87:209-217.
36. Munkvold, G.P., and Stahr, H.M. 1994. Ear rots and mycotoxins in Iowa corn. (Abstr.) Phytopathology 84:1064.
37. Munkvold, G.P., Stahr, H.M., Logrieco, A., Moretti, A., and Ritieni, A. 1998. Occurrence of fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. Appl. Environ. Microbiol. 64:3923-3926.
38. Nash, S.M. and Snyder, W.C. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567-572.
39. Nelson, P.E., Desjardins, A.E., and Plattner, R.D. 1993. Fumonisins, mycotoxins produced by Fusarium species: Biology, chemistry, and significance. Annu. Rev. Phytopathol. 31:233-252.
40. Nelson, P.E., Toussoun, T.A., and Marasas, W.F.O. 1983. *Fusarium* species, an illustrated manual for identification. The Pennsylvania State University Press, University Park and London.
41. Nyvall, R.F. and Kommedahl, T. 1970. Saprophytism and survival of *Fusarium moniliforme* in corn stalks. Phytopathology 60:1233-1235.
42. O'Donnell, K., Cigelnik, E., and Nirenberg, H.I. 1998. Molecular systematics and phylogeny of the *Gibberella fujikuroi* species complex. Mycologia 90(3):465-493.
43. Ojcius, D.M., Zychlinsky, A., Zheng, L.M., and Young, J.D.-E. 1991. Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. Experimental Cell Research 197:43-49.
44. Ooka, J.J. and Kommedahl, T. 1977. Wind and rain dispersal of *Fusarium moniliforme* in corn fields. Phytopathology 67:1023-1026.
45. Oren, L., Ezrati, S., and Sharon, A. 2001. Characterization of *Fusarium*-corn interaction using GFP-transgenic isolate. MPMI Poster Session.Park, J.J., Smalley, E.B., and Chu, F.S. 1996. Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. Appl. Env. Microbiol. 62:1642-1648.
46. Park, J.J., Smalley, E.B., and Chu, F.S. 1996. Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. Appl. Env. Microbiol. 62:1642-1648.
47. Quaedvlieg, N.E.M., Schlaman, H.R.M., Admiraal, P.C., Wijting, S.E., Stougaard, J., and Spaink, H.P. 1998. Fusions between green fluorescent protein and β–glucuronidase as a sensitive and vital bifunctional reporters in plants. Plant Mol. Biol. 37:715-727.
48. Ritchie, S.W., Hanway, J.J., and Benson, G.O. 1997. How a corn plant develops. Iowa State University. Special Report 48.

49. Ritieni, A., Maria, S.M., Randazzo, G., Logrieco, A., Moretti, A., Peluso, G., Ferracane, R., and Fogliano, V. 1997. Teratogenic effects of fusaproliferin on chicken embryos. J. Agric. Food Chem. 46:3039-3043.
50. Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., and van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. Phytopathology 82:353-357.
51. Ross, P.F., Nelson, P.E., Richard, J.L., Osweiler, G.D., Rice, L.G., Plattner, R.D., and Wilson, T.M. 1990. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. Appl. Environ. Microbiol. 56:3225-3226.
52. Sobek, E.A. and Munkvold, G.P. 1999. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. J. Econ. Entol. 92(3):503-509.
53. Sumner, D.R. 1968. Ecology of corn stalk rot in Nebraska. Phytopathology 58:755-760.
54. Turgeon, B.G., Garber, R.C., and Yoder, O.C. 1985. Transformation of the fungal maize pathogen *Cochliobolus heterostrophus* using the *Aspergillus nidulans amdS* gene. Mol. Gen. Genet. 201:450-453.
55. Windels, C.E., Burnes, P.A., and Kommedahl, T. 1988. Five-year preservation of *Fusarium* species on silica gel and soil. Phytopathology 78:107-109.
56. Vigier, B., Reid, L.M., Seifert, K.A., Stewart, D.W., and Hamilton, R.I. 1997. Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario. Can. J. Plant Pathol. 19:60-65.

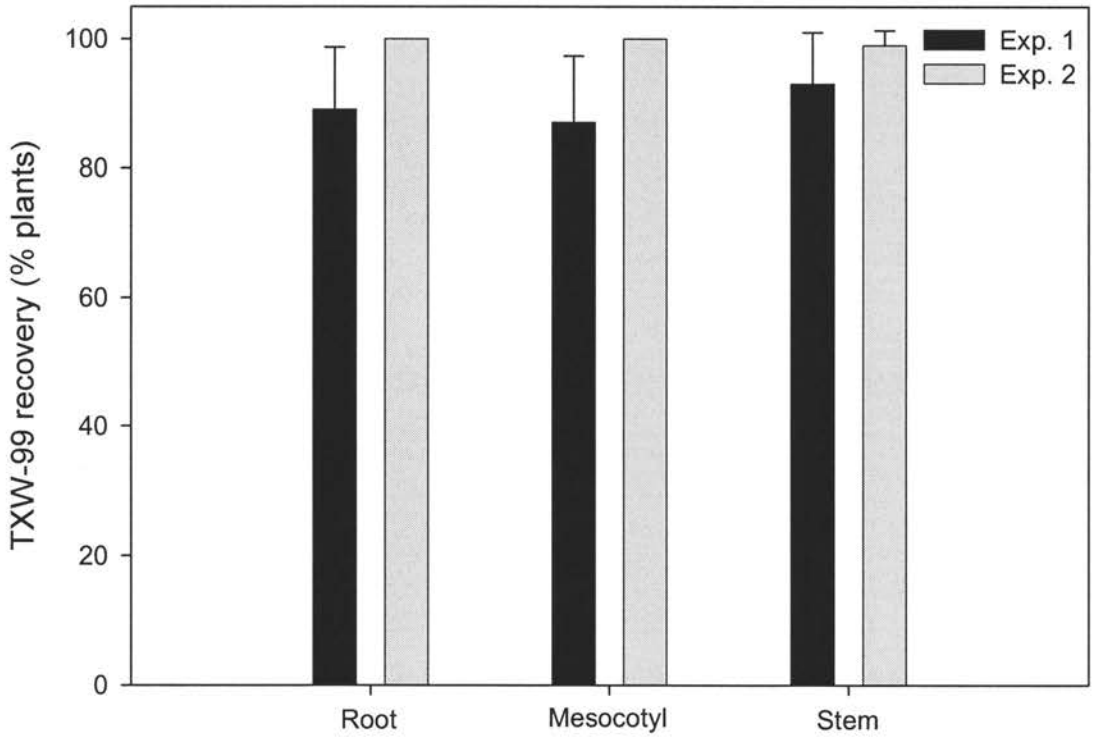

Figure 2.1. Recovery of *F. subglutinans* strain TXW-99 from tissues of maize plants grown from inoculated seeds, sampled at V2. Each value represents the mean of three replications of 8 to 10 plants each. Error bars are standard deviations.

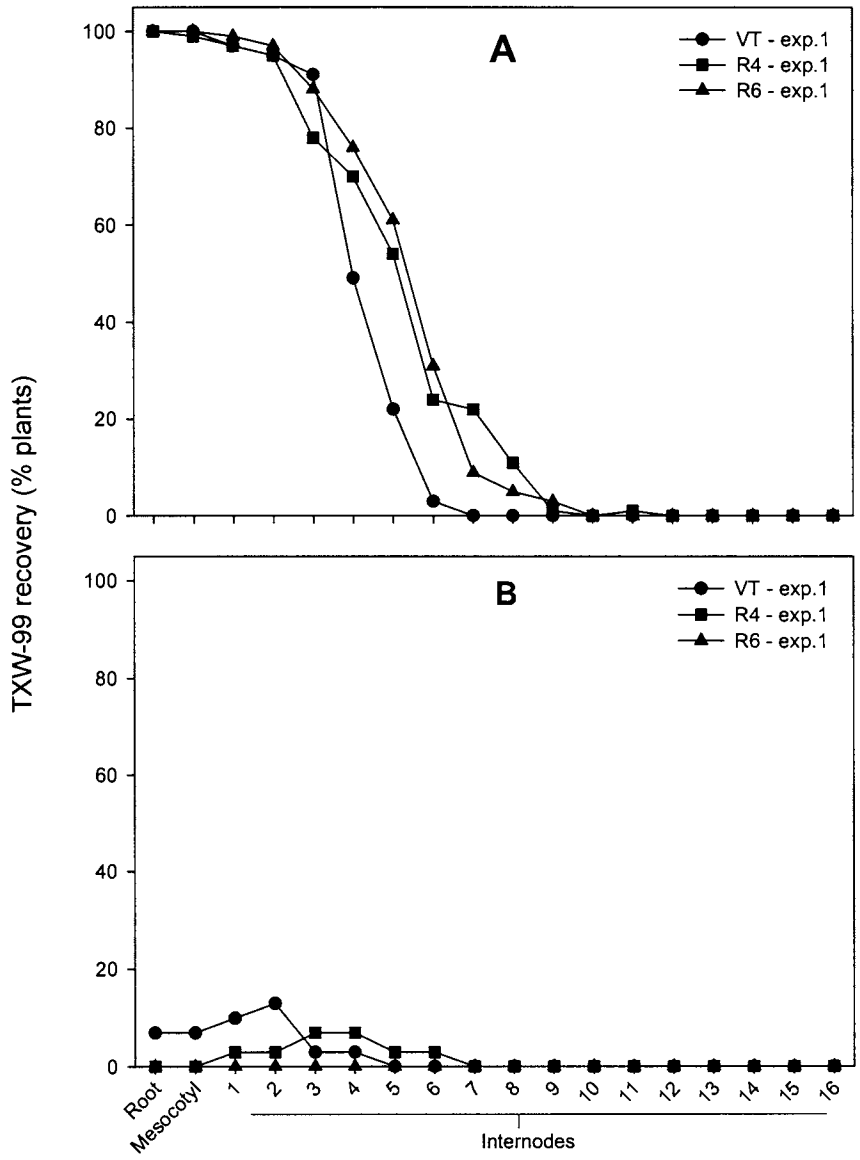

Figure 2.2. Recovery of *F. subglutinans* strain TXW-99 from the root, mesocotyl, and internodes of maize plants grown from inoculated (**A**) and non-inoculated (**B**) seeds, sampled in experiment 1 at VT, R4, and R6. Ears usually were associated with the 9th node. Each value for **A** represents the mean of three replications of 23 to 25 plants each up to the 10th internode.

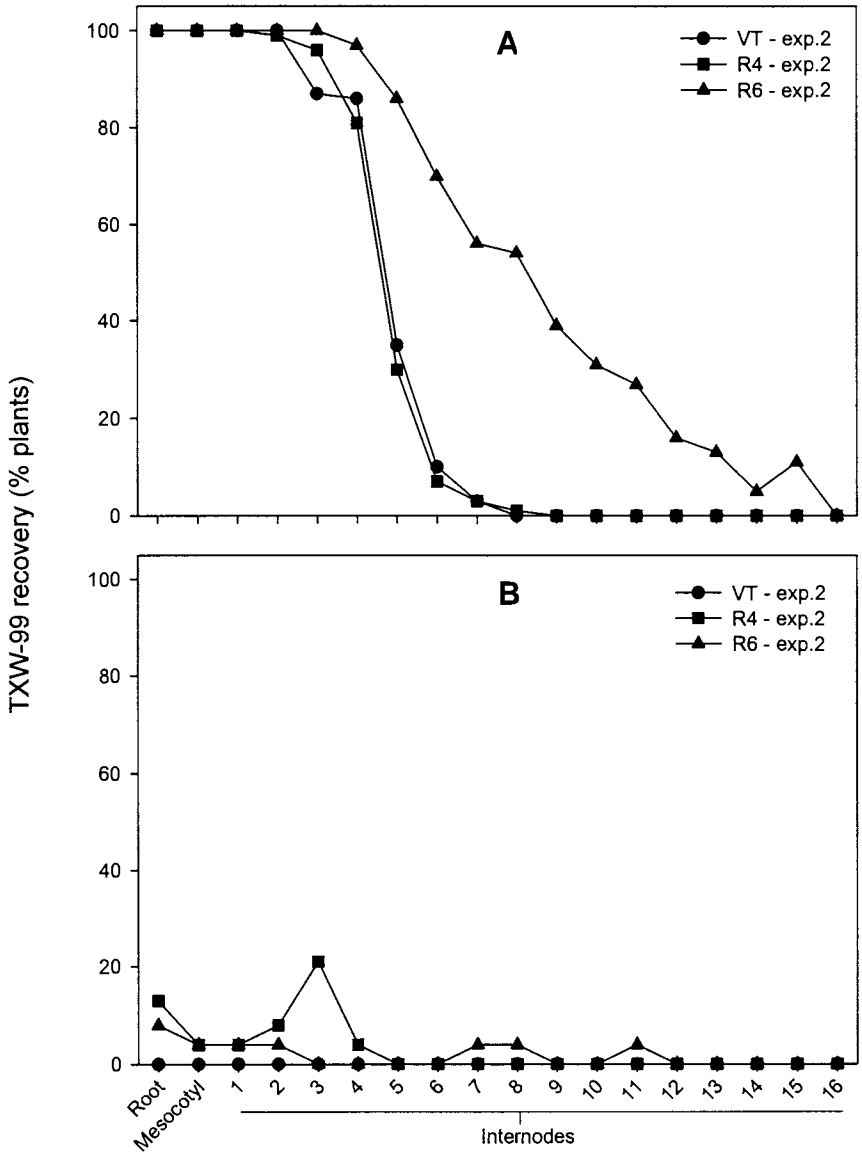

Figure 2.3. Recovery of *F. subglutinans* strain TXW-99 from the root, mesocotyl, and internodes of maize plants grown from inoculated (**A**) and non-inoculated (**B**) seeds, sampled in experiment 2 at VT, R4, and R6. Ears usually were associated with the 9th node. Each value for **A** represents the mean of three replications of 23 to 25 plants each up to the 10th internode at VT and R4 and 14 to 17 plants each up to the 13th internode at R6.

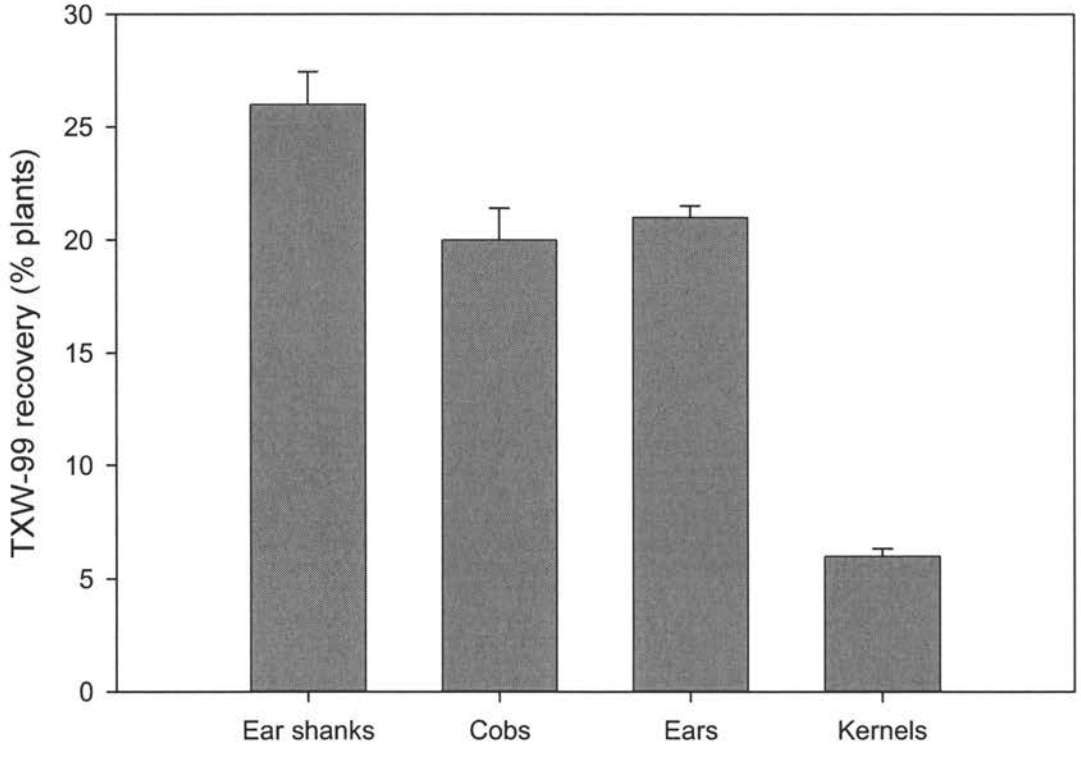

Figure 2.4. Recovery of *F. subglutinans* strain TXW-99 from reproductive tissues of plants grown from inoculated seeds, sampled in experiment 2 at R6. Each value represents three replications of 23 to 24 tissues each for the ear shanks, cobs, and ears and 225 to 232 kernels each. Value for ears represents the mean percentage of ears in which TXW-99 was recovered from at least one kernel. Error bars are standard deviations.

CHAPTER THREE

SEED TRANSMISSION OF *FUSARIUM VERTICILLIOIDES* IN MAIZE PLANTS GROWN UNDER THREE DIFFERENT TEMPERATURE REGIMES

A paper to be submitted to Plant Disease

Amy L. Wilke[1], Charlotte R. Bronson[2], Adriana Tomas[3] and Gary P. Munkvold[4]

Abstract

Fusarium verticillioides is one of the most common fungal pathogens of maize causing root rot, stalk rot, ear rot, seedling blight, and mycotoxin contamination of grain. *F. verticillioides* has been shown to be seed transmitted and cause systemic infection of maize but the frequency of seed transmission and systemic infection has varied widely among and within individual studies. In order to better understand variability in the frequency of systemic infection, we evaluated the effect of temperature on the first step in the systemic infection process, the transmission of *F. verticillioides* from seed to seedling. Seeds of a commercial maize hybrid were inoculated with a strain of *F. verticillioides*, which had been transformed with a gene for green fluorescent protein (GFP). The seeds were planted in a greenhouse potting mix and incubated in growth chambers. The chambers were set to one of

[1]Graduate Research Assistant, Dept. of Plant Pathology, Iowa State University, Ames, Iowa 50011

[2]Professor, Dept. of Plant Pathology, Iowa State University, Ames, Iowa 50011

[3]Senior Research Biologist, Dupont Crop Genetics Research and Development, Wilmington, Delaware 19880

[4]Associate Professor, Dept. of Plant Pathology, Iowa State University, Ames, Iowa 50011

three temperature regimes that were designed to simulate average and extreme temperatures that occur in Iowa during the weeks following planting. Extreme temperatures, both low and high, were determined by calculating two standard deviations below and two standard deviations above the average temperatures. The regimes included day temperatures for a 14-hour light period and night temperatures for a 10-hour dark period. The temperatures of the growth chambers were changed weekly to follow seasonal temperature progression. Plants were sampled at growth stages V2 and V6. The root, mesocotyl, and stem tissues were surface-disinfested and cultured on a semi-selective medium. Fungal growth from the tissues was checked for GFP expression by examination under a fluorescence microscope. At V2, the percentages of infected tissues at the low, average, and high temperature regimes were ≥72%, ≥68%, and ≥75% respectively. At V6, infection of below-ground tissues persisted, and plants had symptomless systemic infection of the above-ground internodes. Percentages of infected internodes above ground (fourth, fifth, and sixth internodes) for the low temperature regime were 24%, 6%, and 3%; for the average temperature regime they were 35%, 9%, and 0%; and for the high temperature regime they were 46%, 24%, and 9%. Seed transmission and systemic infection occurred at all temperatures and did not differ significantly among temperature treatments. These results indicate that seed transmission of *F. verticillioides* is a common event and that symptomless systemic infection can be initiated under a broad range of temperature conditions.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (synonym *F. moniliforme* Sheldon), *F. subglutinans* (Wollenw & Reinking) Nelson, Toussoun, and Marasas, and *F. proliferatum*

(Matsushima) Nirenberg are morphologically similar species and are among the most common *Fusarium* species infecting maize in most areas of the world. All three species cause identical symptoms in maize (*Zea mays* L.) including root rot, stalk rot, ear rot, and seedling blight. These species also generate toxic secondary metabolites (mycotoxins) that can be produced in symptomatic and symptomless plant tissues (16, 17).

The fumonisins, produced by *F. verticillioides* and *F. proliferatum*, are the most frequently occurring class of mycotoxins found in maize kernels. The most common fumonisins are FB_1, FB_2, and FB_3 (16), but there are numerous others. Fumonisins have been proven to cause leukoencephalomalacia (LEM) in horses (25, 32), porcine pulmonary edema (PPE) in swine (25, 32), and are associated with elevated rates of esophogeal cancer in humans (16, 30).

F. verticillioides, *F. subglutinans*, and *F. proliferatum* mycelia survive in maize residues (4) and in seed. Seedborne *F. verticillioides* can be transmitted to seedlings and can be found in symptomless tissues throughout the plant. In 1962, Foley found that *F. verticillioides* (reported as *F. moniliforme*) survives in the pedicel and abscission layers of the seed. Foley (10) and Sumner (35) determined that hyphae grow out of seeds and into the roots, mesocotyls, and stalks of the new plants. They used the term systemic infection to describe this symptomless colonization of the plants because they believed that the fungus moved from the roots to the remainder of the plant. Kucharek and Kommedahl (12) disagreed with this theory of seed transmission and systemic infection. They planted seeds from lots with high and low incidences of infection and found that infection of the resulting plants was not significantly different. For many years the uncertain significance of seed transmission and systemic infection was debated (19).

Recent studies have confirmed Foley and Sumner's conclusions that seed transmission and systemic infection from *F. verticillioides*-infected seeds is a source of root and stalk infection (1, 11). In addition, these studies have demonstrated seed-to-kernel transmission of the fungus from the planted seed, through the plant, to the developing kernels (20, 21). Kedera et al. (11) conducted an experiment in which seeds were inoculated in the laboratory with four different strains of *F. verticillioides* and planted in the field. The seed-inoculated strains (identified by vegetative compatibility) were recovered from 65% of the crowns, 34 to 54% of the nodes, 10% of the cobs, and 8% of the kernels.

A study conducted by Desjardins and Plattner (5) supports systemic infection and further suggests that this may contribute to mycotoxin contamination in grain. A strain of *F. verticillioides* that produced only fumonisin B_2 (FB_2) but not FB_1 or FB_3, was applied to seeds at planting. At physiological maturity, fungi were isolated from the kernels and their fumonisin production was characterized. From the plants that were grown from seeds inoculated with the FB_2-producing strain, 47 to 78% (in 1996 and 1997, respectively) of the strains recovered from the kernels produced only FB_2. From these results it was concluded that the FB_2 producing strain systemically infected the plants and produced FB_2 in the kernels.

The frequency of seed transmission and systemic infection have varied widely among and within individual studies. In some experiments, seed transmission and systemic infection did not play a significant role in plant infection (9, 12, 21). In other studies, seed transmission was detected in up to 80 to 90% of the below-ground tissues of the plants, and symptomless systemic infection was detected in up to 50% of the plants at the seedling stage (3, 21, 39). Beyond the seedling stage, seed transmission was detected in up to 65% of the

plant crowns, and symptomless systemic infection was detected in up to 34 to 54% of the stalk tissues and in 8% of the kernels (11). The various studies differed in fungal strains used, inoculation methods, maize genotypes, and/or environmental conditions. Results have varied even in studies conducted with the same fungal strain (20, 21). These results suggest that environmental factors affect the frequency of seed transmission and systemic infection by *F. verticillioides*.

In order to better understand the influence of environmental variability on the frequency of systemic infection, we evaluated the effect of temperature on the first step in the systemic infection process, the transmission of *F. verticillioides* from seed to seedling.

Materials and Methods

A *Fusarium* strain was isolated from Iowa maize kernels and identified as *F. verticillioides* based on morphological characteristics (26, 27). The strain was deposited at the Pennsylvania State University Fusarium Research Center and designated M-8114.

Transformation. *F. verticillioides* strain M-8114 was transformed with genes for green fluorescent protein (GFP) and hygromycin B resistance using plasmid pIGPAPA, generously provided by B. Gillian Turgeon (Cornell University, NY).

Plasmid pIGPAPA contains the gene sGFP attached to a truncated (and therefore constitutive) isocitrate lyase promoter from *Neurospora crassa*. It also includes a hygromycin B phosphotransferase gene (*hygB*) for selection in fungi and an ampicillin resistance gene for selection in *Escherichia coli*. The plasmid was purified using QIAfilter Plasmid Maxi Kit (Qiagen Inc., Valencia, CA) and was linearized with *Bam*HI.

Protocols for preparation of protoplasts were adopted from methods described for *Cochliobolus heterostrophus* by Turgeon et al. (36). Strain M-8114 was grown on complete

medium with xylose (CMX) (14) for 7 to 10 days. A conidial suspension (9.5×10^6 conidia/ml) was prepared in liquid complete medium (CM) (14) and incubated at 30°C for 20 hours on a rotary shaker at 150 rpm. The resulting mycelial mass was pelleted and then re-suspended in 0.7 NaCl solution. The mycelia were then incubated at 30°C for 1.5 hours in enzyme osmoticum (0.1 mg /ml chitinase (Sigma, St. Louis, MO), 7.5 mg driselase/ml (Sigma, St. Louis, MO), and 7.5 mg Novozyme 234/ml (InterSpex Products Inc., Foster City, CA)) (15) to digest the cell walls. Protoplasts were harvested by filtering the enzyme-osmoticum through two layers of cheesecloth and one layer of Nitex (pore size 20 μm, Tetko Inc., Elenstord, N.Y.). The filtrate was centrifuged for 15 min at 1600 × g and then decanted. The pellet was washed by adding 10 ml of cold STC (1.2 M sorbitol, 10mM Tris (pH 7.5), and 50 mM $CaCl_2$) and centrifuged for 15 min. The supernatant was decanted and the pellet was then re-suspended in 1 ml of STC.

Protocols for transformation were the same as described for *C. heterostrophus* by Turgeon et al. (36) except for the use of regeneration medium in place of molten acetamide medium. Thirty μg of linearized plasmid DNA were added to a tube with approximately 3.5×10^6 protoplasts in 100 μl of STC. Three additions of polyethylene glycol (PEG) solution were made every 10 minutes in aliquots of 200 μl, 200 μl, and 800 μl. During these incubation periods the tube was rolled to mix its contents. One ml of STC was added to dilute the PEG solution. The contents of the tube were then added to regeneration medium (0.1% yeast extract, 0.1% casein enzymatic hydrolysate, 1 M sucrose, and 2% agar) and incubated at 30°C for 24 hours to allow protoplasts to regenerate walls and grow into colonies of mycelia. The plates were overlayed with 20 ml of 1% water agar with 100 μg/ml

hygromycin B (Roche, Indianapolis, IN). Colonies that were transformed were resistant to hygromycin B and therefore grew through the overlay in 4 to 5 days.

Plugs from the resulting colonies were transferred to petri dishes of CMX with hygromycin B. Colonies that were not inhibited by the antibiotic were checked for GFP expression using a fluorescence microscope. Thirty-five colonies were confirmed as transformants based on their resistance to hygromycin B and their ability to fluoresce. The transformants were then placed on silica gel for storage at 4°C (39).

Transformant characterization. Three of the transformants were tested to confirm that the gene for GFP was mitotically stable. Two mitotic stability tests were conducted with GFP transformants of *F. verticillioides* strain M-8114: TXI-1, TXI-49, and TXI-79. The transformants were chosen based on their ability to fluoresce and their rapid radial growth on CMX that was similar to the wild type growth rate.

In the first test, sequential transfers were conducted to determine if gene expression was stable. The test was started by placing a single spore of the transformant on the outside edge of a petri dish containing CMX. For each transformant three replicates were made. The petri dishes were incubated at room temperature under fluorescent lighting. When the colony covered the agar surface, a plug of mycelium was taken from the growing margin and transferred to the edge of a new plate of CMX. Four cycles of growth and transfer were done in this manner. Mycelium from the original petri dishes and the sequential transfers was checked for GFP expression using a fluorescence microscope and the results were recorded as positive or negative.

In a second test, stability of GFP expression through sporulation was tested. Spore suspensions (100 spores/ml) of transformants TXI-1, TXI-49, and TXI-79 were prepared and

1 ml of spore suspension was spread on each of 10 petri dishes of CM and sorbose (14) for each transformant. Sorbose was used in the medium to restrict colony size. The dishes were incubated at 23 to 25°C under fluorescent lights for several days until the resulting colonies were approximately 1 to 3 mm in diameter. Each colony was checked for GFP expression using a fluorescence microscope. The number of colonies with GFP expression was recorded.

An experiment was conducted to determine if transformation had affected the growth of the fungus in liquid culture. Spore suspensions ($1.1x10^6$ spores/ml) of TXI-79 (transformant) and M-8114 (wild type) were made. In 125-ml Erlenmeyer flasks, 1 ml of spore suspension was added to 29 ml of liquid CMX. For each strain, nine flasks of medium were inoculated. The flasks were placed on a rotary shaker at 100 to 125 rpm at 23 to 25°C. After 2 weeks, the contents of each flask were vacuum filtered using pre-weighed filter papers. After filtration the filter papers containing mycelium were placed in petri dishes left partly open. All of the petri dishes were placed in a 26°C incubator to dry for 1 week. The filter papers were then weighed and the dry weight of the mycelial mass was calculated.

A test also was conducted to determine if transformation had affected the ability of TXI-79 to colonize maize stalk tissues. Thirty seeds of Cargill hybrid 1077 (Cargill Inc., Minneapolis, MN) were surface disinfested (2 min in 0.5% NaOCl and 0.5 min in sterile distilled water), planted in pots filled with pasteurized soil (1 part peat: 2 parts soil: 1 part perlite), and placed in the greenhouse. At tasseling (VT) the plants were inoculated with either TXI-79 or strain M-8114 (wild type). Spore suspensions ($1x10^6$ spores/ml) of each fungus were made from 7 to 14-day-old cultures 24 hours before the inoculations and kept at 4°C. Corn plants were injected by syringe just above the second leaf node with 0.5 ml of

spore suspension. Ten plants were inoculated with TXI-79 and five plants with strain M-8114. The stalk of each plant was harvested 46 days after inoculation. The stalks were cut into 30-cm pieces and disinfested for 3 min in 70% ethanol, 5 min in 0.5% NaOCl, and 2 min in sterile distilled water. Internodes were numbered and the center 2 cm portion of each internode (starting just above the second leaf node) was excised from the stalk, split longitudinally, and placed inner surface down on the medium. Tissues of plants that were inoculated with M-8114 were placed on Nash-Snyder medium (24) without PCNB. The tissues of plants inoculated with TXI-79 were placed on Nash-Snyder medium without PCNB and with 100 μl of hygromycin B per liter of medium. Incorporating hygromycin B made the medium semi-selective for the transformants but preliminary experiments indicated that the combination of this antibiotic with PCNB was too inhibitory. The dishes were incubated at room temperature in the dark for 4 to 5 days to allow fungal mycelium to grow out of the tissues. Colonies were checked for the appropriate morphological characteristics and/or GFP expression.

Growth chamber experiments. Several experiments were conducted in growth chambers to assess the effect of temperature on seed transmission and systemic infection of maize by *F. verticillioides*. In one set of experiments (three repetitions of each treatment), plants were grown to stage V2 and in a second set of experiments, plants were grown to stage V6 (31) (also three repetitions per treatment). In each experiment, maize plants were grown from seeds inoculated with strain TXI-79 and seeds that were not inoculated. A spore suspension ($1x10^6$ spores/ml) was made from 7 to 14-day-old cultures of TXI-79 grown on CMX and kept at 4^0C for 10 hours or less until it was used as inoculum. Seeds of Cargill hybrid 1077 were surface disinfested (2 min in 0.5% NaOCl and 0.5 min in sterile distilled

water), placed on sterile paper towels, and allowed to dry in a laminar flow hood. After the seeds were dry they were placed in 125-ml Erlenmeyer flasks, 15 to 25 seeds per flask, with 25 to 50 ml of spore suspension or sterile distilled water. The flasks were placed on a rotary shaker at 100 to 125 rpm for 12 hours. The inoculated and non-inoculated seeds were then placed on sterile paper towels and allowed to dry in separate hoods (one biological safety cabinet and the other a laminar flow).

Each time seeds were inoculated, a sample of 50 inoculated seeds was tested for infestation and infection by TXI-79. Twenty-five of these seeds were placed on Nash-Snyder medium without PCNB and with 100 µl of hygromycin B per liter of medium. The other 25 seeds were surface disinfested in 70% ethanol for 3 min, 10% bleach for 5 min, and sterile distilled water for 2 min and placed on the same medium. The plates were then incubated in the dark at 23 to 25°C for 4 to 5 days. Fungal mycelium was checked for GFP expression and the number of seeds yielding TXI-79 was recorded.

After the seeds had dried they were planted in pots filled with pasteurized soil (1 part vermiculite: 2 parts soil: 1 part peat by volume). For V2 experiments, 35 inoculated seeds and 15 non-inoculated seeds were planted in 4" pots and for V6 experiments, 25 inoculated seeds and 10 non-inoculated seeds were planted in 6" pots. Pots containing inoculated and non-inoculated seeds were then arbitrarily arranged in the growth chamber.

Treatments consist of three temperature regimes (low, average, and high) that were designed to simulate the range of temperature conditions that may occur in Iowa during the weeks following planting. Results of a temperature study conducted over a ten-year period in Ames, Iowa were used to determine the average weekly, day and night temperatures (8) during the growing season starting in early May when the average night temperature was

10°C (adequate for maize germination and growth). This was designated the average temperature regime for our experiments. High and low temperature regimes were determined by calculating two standard deviations above or two standard deviations below the temperatures of the average regime. Each regime involved a weekly progression of day/night temperatures corresponding to the seasonal temperature progression (Figure 3.1). The growth chambers were set for 14-hour light periods (a combination of incandescent and fluorescent light) during which time the day temperatures were used and 10-hour dark periods when the night temperatures were used. Light intensities in the growth chambers range from 98 to 352 μmol $m^{-2}s^{-1}$. Plants were watered as needed throughout the experiment and supplemental fertilizer was not used. Three growth chambers were used for the V2 experiments that were done in triplicate for each temperature regime using a different growth chamber each time. Several growth chambers were used for the V6 experiments that were also done in triplicate using a different growth chamber each time. Data loggers were used to record the actual air temperatures inside the chambers while the experiments were in progress.

At the appropriate sampling date (either V2 or V6) whole plants were harvested, surface disinfested (3 min in 70% ethanol, 5 min in 0.5% NaOCl, and 2 min in sterile distilled water), and dissected. For the V2 sampling date the mesocotyl and stem (portion of the plant between the mesocotyl and the first leaf collar) were measured. Then the seed and primary root (approximately five 2-cm pieces) were placed on Nash-Snyder medium without PCNB and with hygromycin B. The mesocotyl and stem were cut longitudinally and the inner surfaces were directly placed on the medium. For the V6 sampling date, the plants were surface disinfested and measured from the soil surface to the sixth leaf collar. Then the

primary root and mesocotyl of the plants were sampled as described above. The internodes of the plants were also sampled by excising the center portions (1 to 2 cm) and cutting them longitudinally. The inner surfaces of the internodes were directly placed on the medium. Petri dishes were placed in clear, plastic containers and kept in the dark at 23 to 25°C. After several days, the tissues were checked for fungal growth that had GFP expression using a Leitz Fluovert inverted fluorescence microscope.

Data generated from the growth chamber experiments sampled at V2 and V6 were analyzed to determine if temperature affected the ability of TXI-79 to infect maize tissues (analysis of variance using PROC GLM of statistical program SAS version 6.12). For the V2 experiments, a Latin Square analysis was used to determine the effects of chamber sequence (the order that each temperature regime was used in each chamber), growth chamber (growth chamber RSE9, RSE11, or RSE7), and temperature. Analysis for the V2 sampling dates was conducted for each tissue. The V6 experiments could not be analyzed with a Latin Square analysis because more than three growth chambers were used. For these experiments, the analysis was conducted as a 2-way analysis of variance for the effects of chamber and temperature. For each experimental unit at V6, the mean number of above-ground internodes infected with TXI-79 was calculated and used for the statistical analysis.

Analysis of variance (SAS PROC GLM) was also conducted to determine if chamber sequence, growth chamber, temperature regime, or inoculation affected plant height at V2 or V6.

Results

The mitotic stability results indicated that GFP expression was stable through vegetative growth and sporulation in the transformed *F.verticillioides* strains. In the first

experiment, for each transformant tested (TXI-1, TXI-49, and TXI-79), four out of four consecutive transfers for all three replicates maintained GFP expression. In the second experiment, all 411, 340, and 273 colonies derived from conidia of TXI-1, TXI-49, and TXI-79 respectively, expressed GFP (Table B.1). From these results we concluded that GFP would be an effective marker for *F. verticillioides* strain M-8114.

Transformation did not affect the growth of the strain in liquid culture. There was no significant difference in dry weight between TXI-79 and M-8114 (P=0.2564), indicating that the strains grew at comparable rates (Table B.2).

Transformant TXI-79 and the wild type M-8114 colonized maize stalk tissues at similar rates. A PROC GLM analysis of variance was conducted to compare the highest internode of each plant from which TXI-79 and M-8114 were recovered (Table B.3). There was no significant difference in the extent of maize stalk colonization by the two strains (P=0.1277). From this we concluded that transformation did not effect the ability of the fungus to colonize maize stalk tissues.

Our inoculation method provided us with high levels of both seed infestation and infection. For all of the experiments, 100% seeds that were cultured without surface disinfestation were infested with TXI-79. Fifty to 100% of the seeds that were surface disinfested and cultured were infected with TXI-79.

The temperature regimes caused the plants to develop at different rates so the age of plants at V2 or V6 differed among the temperature treatments. Plants grown to growth stage V2 under the low temperature regime were approximately six weeks old, plants in the average temperature regime were approximately four weeks old, and plants in the high temperature regime were approximately two weeks. At V6, the plants grown under the low

temperature regime, the average regime, and the high regime were approximately 12 weeks, eight weeks, and six weeks old, respectively. The low temperature treatment delayed the emergence of the plants for approximately two weeks. Temperature treatment did not, however, affect the number of plants that emerged or the final plant stand counts nor did inoculation. Plants did not display symptoms of seedling disease.

The mean plant heights at V2 were 3.6 cm for the low temperature regime, 5.3 cm for the average temperature regime, and 5.0 cm for the high temperature regime. Chamber sequence, growth chamber, and inoculation did not affect plant height ($P>0.05$) but temperature regime did ($P=0.0224$). For the V6 sampling date, the mean plant height was 16.8 cm for the low temperature regime, 22.4 cm for the average temperature regime, and 26.0 cm for the high temperature regime. For these experiments, an interaction existed between the temperature regimes and the growth chambers ($P=0.0071$), therefore, conclusions could not be drawn on the effects of growth chamber and temperature on plant height.

At the V2 sampling date, TXI -79 was recovered from root, mesocotyl, and stem tissues of plants grown from inoculated seeds under all three temperature regimes. The recovery of TXI-79 from these tissues was ≥72% at the low temperature regime, ≥68% at the average temperature regime, and ≥75% at the high temperature regime (Fig. 3.2). Recovery of TXI-79 from plant tissues grown from non-inoculated seeds was ≤2%.

Chamber sequence and growth chamber did not significantly affect recovery of the fungus from plant tissues at V2 ($P>0.05$). Similarly, recovery of the fungus did not differ among temperature treatments ($P>0.05$) (Table 3.1).

For the V6 sampling date, the recovery of TXI-79 from the below-ground tissues (internode three and below) was ≥68% at the low temperature regime, ≥71% at the average temperature regime, and ≥87% at the high temperature regime (Fig. 3.3). In the above-ground tissues (internodes ≥4), there was a steep decline in the recovery of TXI-79 from progressively higher internodes for all three treatments and the fungus was not recovered from tissues above the sixth internode. For the plants grown from non-inoculated seeds, TXI-79 was not recovered from the below-ground tissues or the above-ground tissues.

Neither growth chamber nor temperature treatment significantly affected the mean number of infected above-ground internodes (P=0.8608 and P=0.7683, respectively).

Discussion

At growth stages V2 and V6, temperature did not have an effect on the ability of TXI-79 to be seed transmitted and cause systemic infection in maize plants. In our experiments, seed transmission occurred at a high frequency, which is consistent with some previous work (4, 11, 13, 21). Our results also showed TXI-79 resided in the crown tissues of the plants at the early growth stages, which was similar to the work done by Lawrence et al. (13), who also found that the fungus does not colonize the entire plant until after tasseling. Seed-to-kernel transmission involves several stages, and seed-to-seedling transmission is only the first stage (21). Our results suggest that seed-to-seedling transmission is probably not the limiting stage for transmission of the fungus. Apparently this stage of the process occurs at a high frequency under a broad range of temperature conditions. Seed-to-kernel transmission tends to occur at a lower frequency indicating that limiting factors may operate at some later

stage in the process. It is quite possible that temperature and other environmental factors affect the systemic infection process at these later stages.

The temperature treatments affected the growth of the plants and most likely affected the growth of the fungus as well. The optimal temperature for maize plant growth is 30°C (2) and the optimal temperature for *F. moniliforme* growth is 28°C (38) to 30°C (18). The high and average temperature regimes reached these temperatures but the low regime did not. The different temperature regimes, therefore, caused the plants to develop at different rates so plants grown at the low temperature regime were older and shorter than those grown at the average and high temperature regimes when they were sampled. It is possible that because the low temperature plants were shorter that the fungus did not need to grow as extensively to reach a given internode. It is also very likely that fungal growth was slowed in the low temperature treatment but because these plants were older the fungus had more time to colonize the tissues, resulting in a level of infection similar to the warmer temperature treatments. These factors illustrate that temperature effects on the plant-pathogen interaction are more complex than can be predicted by direct effects of temperature on fungal growth.

GFP was a suitable marker for *F. verticillioides* strain M-8114 (transformant TXI-79) and it allowed us to distinguish our strain from other naturally occurring *Fusarium* strains in the environment. GFP also proved to be more reliable and convenient than vegetative compatibility that has been used as a marker in previous studies (4, 11, 20, 21). Unlike GFP, which was maintained during our experiments, vegetative compatibility can be lost if auxotrophic mutants become dysfunctional and can not form heterokaryons or revert back to wild-type growth (4). Obtaining the results of an experiment is also faster and easier with GFP than with vegetative compatibility which is labor intensive.

Maize seeds planted under cool conditions in the field often experience seedling disease problems (33) but we did not observe symptoms in our study. It is likely that the *Pythium* species that primarily cause seedling disease at low temperatures (6) were not present in the pasteurized soil used in this study. The lack of seedling disease can also be attributed to the fact that soil moisture was not excessive in our study. Additionally, there is variability in aggressiveness among *Fusarium* strains (22), and some strains have little capacity to cause seedling blight.

These results indicate that seed transmission of *F. verticillioides* is a common event and that symptomless systemic infection can be initiated under a broad range of temperature conditions. The primary significance of this phenomenon may lie in the contribution of seed-to-kernel transmission to mycotoxin contamination of grain. However, more research is needed in order to define this contribution.

Literature Cited

1. Bacon, C.W. and Hinton, D.M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Can. J. Bot. 74:1195-1202.
2. Blacklow, W.M. 1972. Influence of temperature on germination and elongation of the radicle and shoot of corn (Zea mays L.). Crop Sci. 12:647-650.
3. Cotten, T.K. 1996. Survival and seed transmission of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium subglutinans* in maize. M.S. Thesis. Iowa State University. 75 pp.
4. Cotten, T.K. and Munkvold, G.P. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. Phytopathology 88:550-555.
5. Dejardins, A.E., and Plattner, R.D. 2000. Fumonisin B_1 – nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. J. Agric. Food Chem. 48:5773-5780.
6. Dodd, J.L. and White, D.G. 1999. Seed rot, seedling blight, and damping off pp. 10-11, in Compendium of Corn Diseases, 3rd edition. APS Press, St. Paul.
7. Dowd, P.F. 1998. Involvement of arthropods in the establishment of mycotoxigenic fungi under field conditions. Pp. 307-350 in Mycotoxins in Agriculture and Food Safety, K.K. Sinha and D. Bhatnagar, Eds. Marcel Dekker, Inc. NY.
8. Elford, C.R. and Shaw, R.H. The climate of Iowa: soil temperatures at Ames. 24, 1-70. 1960. Agricultural and Home Economics Experiment State, Iowa State University of Science and Technology. Special Report.
9. El-Meleigi, M.A., Claflin, L.E., and Raney, R.J. 1983. Effect of seedborne *Fusarium monliforme* and irrigation scheduling on colonization of root and stalk tissue, stalk rot incidence, and grain yields. Crop Sci. 23:1025-1028.
10. Foley, D.C. 1962. Systemic infection of corn by *Fusarium moniliforme*. Phytopathology 52:870-873.
11. Kedera, C.J., Leslie, J.F., and Claflin, L.E. 1992. Systemic infection of corn by *Fusarium moniliforme*. (Abstr.) Phytopathology 82:1138.
12. Kucharek, T.A. and Kommedahl, T. 1966. Kernel infection and corn stalk rot caused by *Fusarium moniliforme*. Phytopathology 56:983-984.
13. Lawrence, E.B., Nelson, P.E., and Ayers, J.E. 1981. Histopathology of sweet corn seed and plants infected with *Fusarium moniliforme* and *F. oxysporum*. Phytopathology 71:379-386.
14. Leach, J., Lang, B.R., and Yoder, O.C. 1982. Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*. J. Gen. Microbiol. 128:1719-1729.
15. Lu, S., Lyngholm, L., Yang, G., Bronson, C., and Yoder, O.C. 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated intergration. Proc. Natl. Acad. Sci. 91:12649-12653.
16. Marasas, W.F.O., Miller, J.D., Riley, R.T., and Visconti, A. 2000. Fumonisin B_1. Environmental Health Criteria 219:1-149.
17. Marasas, W.F.O., Nelson, P.E., and Toussoun, T.A. 1984. Toxigenic *Fusarium* species: identity and mycotoxicology. Penn State Univ. Press, State College, PA. pp. 250.

18. Marin, S., Sanchis, V., and Magan, N. 1995. Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. Can. J. Microbiol. 41:1063-1070.
19. McGee, D.C. 1988. Fusarium stalk and ear rot. Pp. 13-14. in .Maize Diseases: A reference source for seed technologists. APS Press, St. Paul.
20. Munkvold, G.P. and Carlton, W.M. 1997. Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds. Plant Dis. 81:211-216.
21. Munkvold, G.P., McGee, D.C., and Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. Phytopathology 87:209-217.
22. Munkvold, G.P. and O'Mara, J.K. 2002. Laboratory and growth chamber evaluation of fungicidal seed treatments for maize seedling blight caused by *Fusarium* species. Plant Dis. 86:143-150.
23. Munkvold, G.P., Stahr, H.M., Logrieco, A., Moretti, A., and Ritieni, A. 1998. Occurrence of fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. Appl. Environ. Microbiol. 64:3923-3926.
24. Nash, S.M. and Snyder, W.C. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567-572.
25. Nelson, P.E., Desjardins, A.E., and Plattner, R.D. 1993. Fumonisins, mycotoxins produced by Fusarium species: Biology, chemistry, and significance. Annu. Rev. Phytopathol. 31:233-252.
26. Nelson, P.E., Toussoun, T.A., and Marasas, W.F.O. 1983. *Fusarium species, an illustrated manual for identification*. The Pennsylvania State University Press, University Park and London.
27. Nirenberg, H.I. 1976. Untersuchugen uber die morphologische Differenzierung in der *Fusarium*-Sektion Liseola. Mitt. Biol. Bundesanst. Land-Forstwirtsch. Berlin-Dahlem 169:1-117.
28. Nyvall, R.F. and Kommedahl, T. 1970. Saprophytism and survival of *Fusarium moniliforme* in corn stalks. Phytopathology 60:1233-1235.
29. Ooka, J.J. and Kommedahl, T. 1977. Wind and rain dispersal of *Fusarium moniliforme* in corn fields. Phytopathology 67:1023-1026.
30. Park, J.J., Smalley, E.B., and Chu, F.S. 1996. Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. Appl. Env. Microbiol. 62:1642-1648.
31. Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., and van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. Phytopathology 82:353-357.
32. Ritchie, S.W., Hanway, J.J., and Benson, G.O. 1997. How a corn plant develops. Iowa State University. Special Report 48.
33. Ross, P.F., Nelson, P.E., Richard, J.L., Osweiler, G.D., Rice, L.G., Plattner, R.D., and Wilson, T.M. 1990. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. Appl. Environ. Microbiol. 56:3225-3226.

34. Smith, D.R. and White, D.G. 1988. Diseases of corn pp. 688-749, in Corn and Corn Improvement, 3rd edition. American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil science Society of America, Inc., Madison.
35. Sobek, E.A. and Munkvold, G.P. 1999. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. J. Econ. Entol. 92(3):503-509.
36. Sumner, D.R. 1968. Ecology of corn stalk rot in Nebraska. Phytopathology 58:755-760.
37. Turgeon, B.G., Garber, R.C., and Yoder, O.C. 1985. Transformation of the fungal maize pathogen *Cochliobolus heterostrophus* using the *Aspergillus nidulans amdS* gene. Mol. Gen. Genet. 201:450-453.
38. Watson, S.A. 1999. Measurement and maintenance of quality. pp. 159 in Corn: Chemistry and Techniques. S.A. Watson and P.E. Ramstead, eds. American Association of Cereal Chemists, Inc., St. Paul.
39. Windels, C.E., Burnes, P.A., and Kommedahl, T. 1988. Five-year preservation of *Fusarium* species on silica gel and soil. Phytopathology 78:107-109.

Table 3.1. Results of analysis of variance for the effects of chamber sequence, growth chamber, and temperature on the recovery of TXI-79 from maize plant tissues sampled at growth stage V2 or V6 in growth chamber experiments.

Growth stage	Tissue	Effect	DF	Mean Square	F Value	P Value
V2	Root	Sequence	2	63.400	5.98	0.1432
		Chamber	2	139.000	13.11	0.0709
		Temperature	2	45.400	4.28	0.1893
	Mesocotyl	Sequence	2	12.400	0.17	0.8558
		Chamber	2	49.000	0.67	0.6003
		Temperature	2	27.400	0.37	0.7287
	Stem	Sequence	2	117.844	1.30	0.4340
		Chamber	2	1269.778	14.05	0.0664
		Temperature	2	74.178	0.82	0.5492
V6	Internodes	Chamber	4	0.127	0.30	0.8608
		Temperature	2	0.125	0.30	0.7683

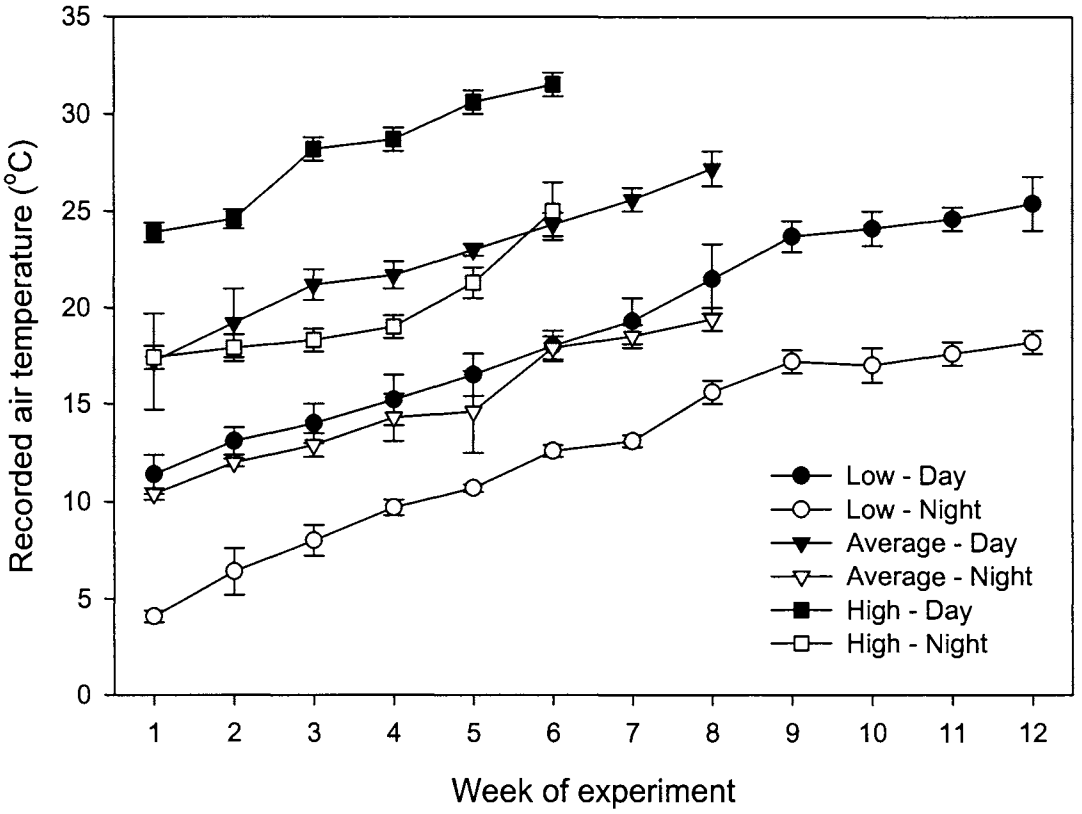

Figure 3.1. Recorded air temperatures for V2 and V6 growth chamber experiments. Temperature regimes were based on average weekly day and night temperatures from a ten year weather study by Elford and Shaw (8). Low and high temperature regimes were determined by calculating two standard deviations below or two standard deviations above the temperatures of the average regime. Error bars are standard deviations, based on mean temperatures for three and six replicated experiments.

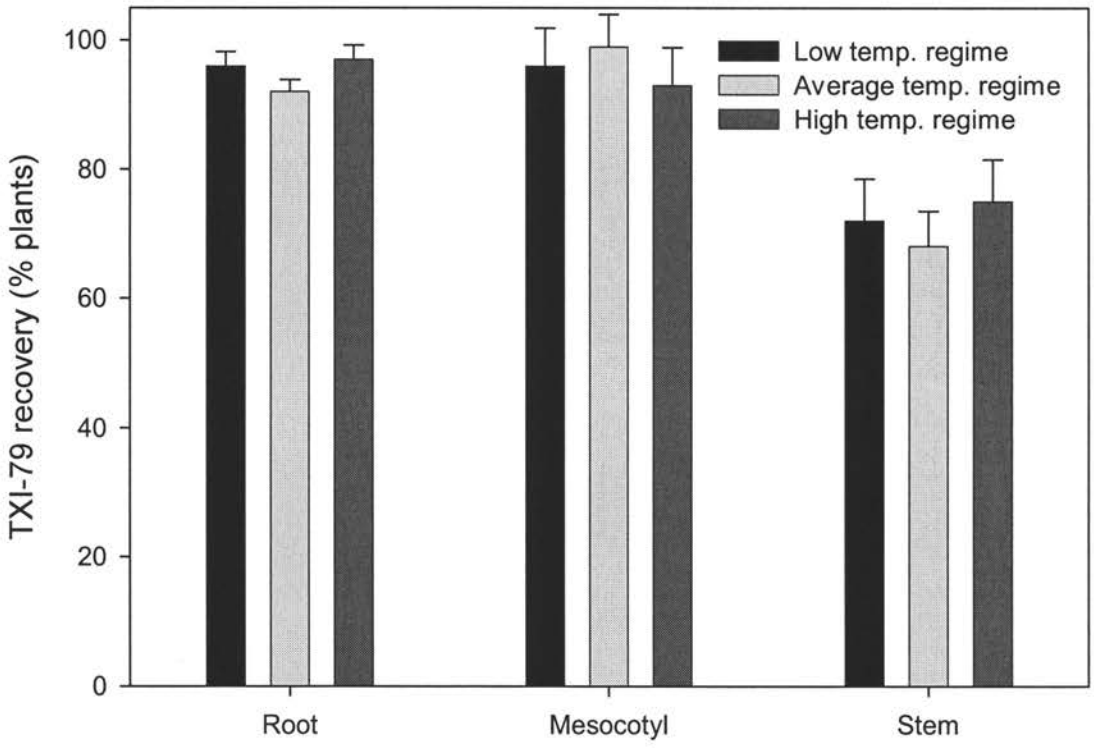

Figure 3.2. Recovery of *F. verticillioides* strain TXI-79 from tissues of V2 maize plants grown from inoculated seeds at different temperatures. Each value represents the mean of three replications of 22 to 35 plants each. Error bars are standard deviations.

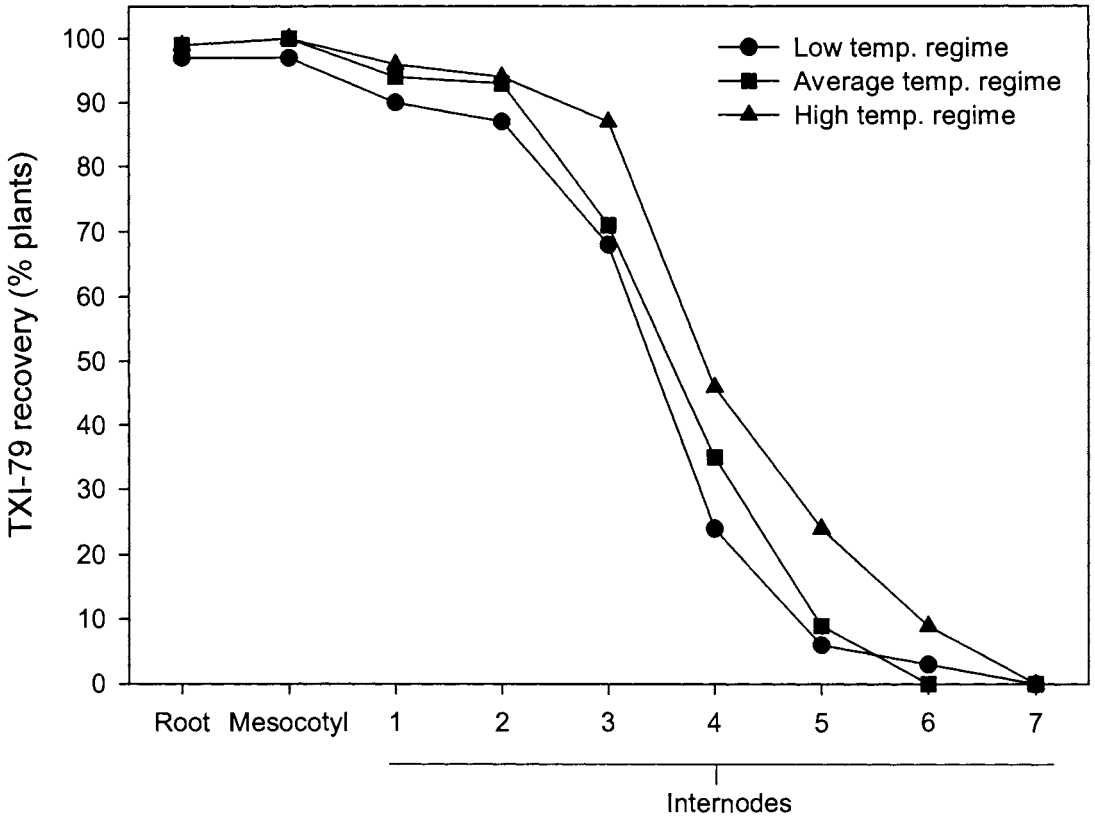

Figure 3.3. Recovery of *F. verticillioides* strain TXI-79 from tissues of V6 maize plants grown from inoculated seeds at different temperatures. Each point represents the mean of three replications of 21 to 25 plants each.

CHAPTER FOUR

GENERAL CONCLUSIONS

Infection and colonization of plant tissues by *Fusarium* species may result in disease or symptomless, systemic infection. Symptomless infection is a concern because mycotoxins are still produced in the affected tissues. Therefore, it is important to understand all of the inoculum sources and pathways of infection for *F. verticillioides*, *F. subglutinans*, and *F. proliferatum*. The objectives for this study were to determine if *F. subglutinans* can be seed transmitted and cause systemic infection in maize and to determine the effects of temperature on the ability of *F. verticillioides* to be seed transmitted and cause systemic infection in maize.

In Chapter 2, we assessed whether or not *F. subglutinans* could be seed transmitted and cause systemic infection in maize. We found that *F. subglutinans* was seed transmitted and caused symptomless, systemic infection. Ninety to one hundred percent of plants grown from TXW-99-inoculated seeds were infected even though seed infection was only 80% in experiment 1 and 88% in experiment 2. This suggests that the infection process of *F. subglutinans* can start from either infected seeds or infested seeds. At the early growth stage of V2, TXW-99 was recovered from the roots and crowns of the plants. During the later growth stages (VT and R4), TXW-99 moved through the stalks of the plants and could be recovered from tissues below the 9^{th} internode. At R6 in experiment 2, TXW-99 was recovered from the entire plant including the ear tissues. In this experiment, TXW-99 also was recovered from 39% of the internodes associated with the ear and recovery from the seeds was 6%. To our knowledge this is the first report of symptomless systemic infection and seed-to-kernel transmission by *F. subglutinans* in maize.

In Chapter 3, we addressed the issue of the affect of temperature on the ability of *F. verticillioides* to be seed transmitted and cause systemic infection in maize. We found that at growth stages V2 and V6, temperature did not have an effect on the frequency of *F. verticillioides* to be seed transmitted and to cause systemic infection in maize plants. In our experiments, seed transmission occurred at a high frequency. Our results also showed that *F. verticillioides* resided in the crown tissues of the plants at early growth stages. Seed-to-kernel transmission involves several stages and seed-to-seedling transmission is probably not the limiting stage. Apparently this stage of the process occurs at a high frequency under a broad range of temperature conditions. Seed-to-kernel transmission tends to occur at a lower frequency indicating that limiting factors may operate at some later stage in the process. The effects of temperature and other environmental factors on the systemic infection process at these later stages need to be investigated.

APPENDIX A

Table A.1. Number of *F. subglutinans* transformant (TXW-4, TXW-36, and TXW-99) colonies checked for GFP expression, the number with expression, and the resulting percent.

Transformant	# of colonies	# with GFP expression	% with GFP expression
TXW-4	1086	1086	100
TXW-36	1130	1130	100
TXW-99	828	828	100

Table A.2. Mycelial dry weights from liquid shake cultures of *F. subglutinans* wild type ITEM2284 and tranformant TXW-99.

Strain	Replicate	Dry Weight (g)
ITEM2284	1	0.0878
ITEM2284	2	0.0872
ITEM2284	3	0.0824
ITEM2284	4	0.0893
ITEM2284	5	0.0788
ITEM2284	6	0.0804
ITEM2284	7	0.0648
ITEM2284	8	0.0838
ITEM2284	9	0.0787
TXW-99	1	0.1161
TXW-99	2	0.0856
TXW-99	3	0.1087
TXW-99	4	0.0893
TXW-99	5	0.1127
TXW-99	6	0.1118
TXW-99	7	0.0902
TXW-99	8	0.1150

Table A.3. Recovery of *F. subglutinans* wild type ITEM2284 and transformant TXW-99 from maize stalk tissues sampled 46 days after inoculation at VT.

		Internodes													
Strain	Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ITEM2284	1	+	+	+	-	-	-	-	-	-	-	-	-	-	
ITEM2284	2	+	+	+	-	-	-	-	-	-	-	-	-		
ITEM2284	3	+	+	+	+	+	+	-	-	-	-	-			
ITEM2284	4	+	+	+	+	+	-	-	-	-	-	-	-		
ITEM2284	5	+	+	+	+	-	-	-	-	-	-	-	-		
TXW-99	1	+	+	+	-	-	-	-	-	-	-	-	-	-	
TXW-99	2	+	+	+	-	-	-	-	-	-	-				
TXW-99	3	+	+	+	+	+	-	-	-	-	-	-			
TXW-99	4	+	-	-	-	-	-	-	-	-	-				
TXW-99	5	+	+	+	-	-	-	-	-	-	-	-			
TXW-99	6	+	+	+	-	-	-	-	-	-	-	-			
TXW-99	7	+	+	+	+	-	-	-	-	-	-	-	-	-	
TXW-99	8	+	+	+	+	-	-	-	-	-	-	-			
TXW-99	9	+	+	+	+	-	-	-	-	-	-	-	-	-	
TXW-99	10	+	+	+	-	-	-	-	-	-	-	-	-		

APPENDIX B

Table B.1. Number of *F. verticillioides* transformant (TXI-1, TXI-49, and TXI-79) colonies checked for GFP expression, the number with expression, and the resulting percent.

Transformant	# of colonies	# with GFP expression	% with GFP expression
TXI-1	411	411	100
TXI-49	340	340	100
TXI-79	273	273	100

Table B.2. Mycelial dry weights from liquid shake cultures of *F. verticillioides* wild type M-8114 and transformant TXI-79.

Strain	Replicate	Dry weight (g)
M-8114	1	0.0980
M-8114	2	0.0934
M-8114	3	0.0973
M-8114	4	0.0656
M-8114	5	0.0688
M-8114	6	0.0873
M-8114	7	0.0825
M-8114	8	0.0629
M-8114	9	0.0848
TXI-79	1	0.0940
TXI-79	2	0.0990
TXI-79	3	0.1074
TXI-79	4	0.0799
TXI-79	5	0.0266
TXI-79	6	0.1151
TXI-79	7	0.0957

Table B.3. Recovery of *F. verticillioides* wild type M-8114 and transformant TXI-79 from maize stalk tissues sampled 46 days after inoculation at VT.

		Internodes													
Strain	Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M-8114	1	+	+	-	-	-	-	-	-	-	-	-			
M-8114	2	+	+	+	+	-	-	-	-	-	-	-	-		
M-8114	3	+	+	+	-	-	-	-	-	-	-	-	-	-	
M-8114	4	+	+	+	-	-	-	-	-	-	-	-			
M-8114	5	+	+	+	-	-	-	-	-	-	-	-	-		
TXI-79	1	+	+	+	-	-	-	-	-	-					
TXI-79	2	+	+	+	-	-	-	-	-	-	-	-	-		
TXI-79	3	+	+	+	-	-	-	-	-	-	-	-			
TXI-79	4	+	+	+	-	-	-	-	-	-	-	-			
TXI-79	5	+	+	-	-	-	-	-	-	-	-	-			
TXI-79	6	+	+	+	-	-	-	-	-	-	-	-	-	-	-
TXI-79	7	+	+	+	+	-	-	-	-	-	-	-			
TXI-79	8	+	+	-	-	-	-	-	-	-	-	-	-		
TXI-79	9	+	+	+	-	-	-	-	-	-	-	-			
TXI-79	10	+	+	-	-	-	-	-	-	-	-				

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Gary Munkvold, for the opportunity to work on this project. Thank you also to Dr. Munkvold and the members of my committee, Dr. Charlotte Bronson, Dr. Adriana Tomas, and Dr. Manjit Misra, for your guidance and support. I would further like to thank Dr. Bronson for her time and patience in answering my endless questions. To Jennifer Perry and Dave Volkers, thank you for your contributions to my research and for being my friends. To all of the friends I have made here, thank you for your laughter, encouragement, and the memories we have made.

To my family and friends, thank you for always supporting me in whatever I do. Thank you also for putting up with the hysterical late night phone calls and crazy emails. Most of all I want to thank you all for believing in me more than I believed in myself and for making everything in my life seem possible.